NORMAN Working Group on Wastewater Reuse
WG 5

Mandate

SUMMARY

Urban wastewater reuse is considered globally as the most critical element of sustainable water management. Water scarcity, foreseen to aggravate by climate change, pushes for maximum utilisation of non-conventional water to balance the increased demand.

The reuse of water in the urban environment is driven by different motivations in the various parts of the world. The European Mediterranean countries suffer, for example, from prolonged dry periods, while the Northern European countries, even though they possess adequate quantities of fresh water they aim to a smaller footprint with regard to the water extraction and reduced energy consumption in relation to water production. On an international level, the fast development of mega-cities in high-growth areas like Asia and South America aggravates urban water consumption and increases the pressure towards solutions to balance the water demand.

Although reuse is accompanied by various benefits, a number of questions still puzzle scientists. The applied treatment fails to remove completely micropollutants and antibiotic resistant bacteria and/or genes. Knowledge on the actual effects of the reuse practice with regard to these aspects is not consolidated.

WG5 aims at answering critical related questions, to provide insight on the effects of long-term exposure of organisms including those at the sub-lethal level, consolidate data on crops uptake, establish criteria/specs on technologies/assessment methods, and suggest new effluent quality criteria to overcome current barriers and enhance further the reuse.

INTRODUCTION/BACKGROUND

In response to the escalating problem of water shortage, treated wastewater is nowadays widely reused and is generally considered as a reliable alternative water source for irrigation and replenishment among other applications. Water demands already exceed supplies in regions with more than 40% of the world's population and in just 12 years as much as 60% of the world's people may confront water scarcity. Reuse in the urban environment is applied in worldwide, driven however by different motivations. Although the reuse practice is accompanied by a number of benefits relating to the enhancement of water balance and soil nutrition, a number of unanswered questions are still related to this practice. Besides the lack of knowledge in respect to possible elemental interactions that may influence the accumulation of metals/elements in the soil and the subsequent uptake by plants and crops, organic micropollutants and antibiotic-resistant bacteria and genes (ARB&Gs) in treated wastewater require much attention. The effluents' remaining organic matter after conventional treatment consists of a number of recalcitrant organic compounds including potential endocrine disrupting compounds, antibiotics and others as the currently applied treatment processes fail to remove completely such contaminants including ARB&Gs, and this leads to their subsequent release in the terrestrial and aquatic environment through disposal and reuse applications which is of major environmental and health concern.

Current open challenges include the contaminants of emerging concern, their physicochemical and biological transformation products originating both during treatment and while being in the environment, their potential uptake by plants/crops, the effects that these contaminants may induce in the environment, the evolution and release of antibiotic

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resistance, the identification of technologies that are able to remove such contaminants from wastewater, and the identification of means and solutions to overcome these problems and promote safe reuse practices further. To avoid negative environmental and human impacts, regulatory frameworks are required, based on validated scientific information. WG5 aims at increasing the scientific understanding on these crucial issues and to potentially boost technological developments.

PROPOSED MANDATE

The WG will focus its efforts on:

1) Revealing the role of wastewater reuse in the evolution and spread of antibiotic resistance
2) Conducting studies and consolidating information on the contaminants uptake by crops
3) Revealing and counteracting weaknesses/knowledge gaps in environmental chemistry and microbiology/toxicology required for the abovementioned activities
4) Delivering best-practice advice to practitioners and dissemination of unbiased perspectives of scientific knowledge to decision makers and the public
5) Assessing which of the information is valid and reliable to be used in regulatory frameworks (e.g. Water Framework and Urban Wastewater Treatment Directive)

The following sub-working groups (SWGs) will be formulated:

SWG1: Microbiome and Mobile Resistome present in combined treatment and reuse systems
SWG2: Uptake and Transmission of Micropollutants and ARB&Gs by Crops
SWG3: Technologies Efficient and Economically Viable against the Current Urban Wastewater Reuse Challenges
SWG4: Risk assessment and policy development

WG5 aims at establishing a strong scientific collaboration channel with the existing WG3 of the NORMAN network on 'Effect-direct analysis for hazardous pollutant identification and WG2 on 'The value of bioassays and biomarkers in water quality monitoring programmes: strategies for the interpretation of results' so that valuable insight already produced will be utilised in the activities related to the wastewater reuse. The same applies for WG1 on the 'Prioritisation of emerging substances'.

More details on the suggested activities of the various SWGs are provided below:

SWG1: Microbiome and Mobile Resistome present in combined treatment and reuse systems

Based on the fact that antibiotic resistance bacteria and genes (ARB&Gs) should be considered as contaminants of emerging concern, information on the capacity of the currently applied wastewater treatment systems in removing antibiotic resistance will be gathered and evaluated. Model sites in various countries will be examined in relation to wastewater reuse implemented schemes and selected antibiotic resistance determinants and mobile genetic elements in the final effluents.

SWG2: Uptake and Transmission of Micropollutants and ARB&Gs by Crops

The uptake of emerging contaminants by crops during wastewater reuse applications has been proved in many cases. A synthesis of knowledge however is required so as to be able to reach concrete conclusions, and develop relevant guidelines. More systematic studies under real environmental conditions are required and relevant suggestions will be elaborated.

SWG3: Technologies Efficient and Economically Viable against the Current Urban Wastewater Reuse Challenges

Even in the countries that have established effluent quality criteria for reuse practices, these do not refer to micropollutants and antibiotic resistance determinants. The standards followed lack behind technological advancements and it is considered as highly important to look thoroughly into the technologies available (including innovative and economically viable ones) so as to be able to progress the state-of-the-art with regard to the standards followed.

Synthesis on the information on the effects of the inadvertent chronic exposure of organisms to micropollutants is currently absent and highly required. Moreover, long-term effects of micropollutants and ARB&Gs need to be assessed and understood. The tests/methods for assessing their adverse effects in wastewater are not standardised (i.e. validated protocols are required). Based on the fact that the reuse applications should integrate phytoxicity/ecotoxicity tests, a collection of the information available will be carried out with the aim to develop a white paper on what is required for wastewater reuse schemes.

The operation cost of such technologies needs to be determined so that comparison of technologies could be feasible taking into account their efficiency in removing the aforementioned compounds. The aim is to develop a white paper on the various technologies and their capacity towards the mentioned contaminants removal.

SWG4: Risk assessment and policy development
To facilitate decision-makers to progress the standing situation with regard to practices and quality criteria applied and overcome existing problems and barriers, a risk assessment framework will be developed in order to facilitate the policy development and prepare suggestions for annexes of the existing European Council Directives such as the Water Framework and Urban Wastewater Treatment Directive, etc.

OUTCOMES OF YEAR 2013

1) Report on the workshop entitled 'Wastewater Reuse Applications and Contaminants of Emerging Concern', organised in Cyprus in September 2012 (already submitted to NORMAN network).

2) Existing White Paper (Mandate) on the WG5 activities, based on decisions taken during the kick-off meeting of WG5 held in Vienna, Austria in June 2013.

3) Road map of activities for 2014 (See Annex I).

WORKPLAN / ROADMAP FOR 2014

A detailed description of the proposed activities are provided in Annex I.

ORGANISATION

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WORKING GROUP EXPERTS

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It is noted that interest in the activities of WG5, has already been expressed by the DG Joint Research Centre (Unit H 01 - Water Resources Unit) of the European Commission (Bernd Manfred Gawlik, Action Leader and Laura Alcalda Sanz).

ANNEX I

Road Map of Activities for 2014

Title of Proposed Activity:

MOBILE ANTIBIOTIC RESISTOME IN WASTEWATER AROUND EUROPE

INTRODUCTION

Antibiotic resistance prevalence has increased worldwide over the last decades with dramatic consequences for human health. This situation is in part due to the excessive use of antibiotics. Because hospitals may discharge their effluents into municipal collectors, and palliative care and patient recovery is increasingly made at home, municipal sewage treatment systems are considered one of the most important hotspots for resistance evolution in urban areas. On average, a municipal wastewater treatment plant discharges billions of antibiotic resistant bacteria (>10^{12}) per day to the surrounding environment, facilitating horizontal transmission of mobile antibiotic resistance genes to other bacteria thriving in soil and water environments.

This problem is amplified, because in an increasing number of countries water scarcity is becoming a reality. Water reuse is regarded nowadays as an unavoidable practice for sustainable management of this natural resource. Despite the benefits of water reuse, at this stage it is difficult to assess in which side the balance tilts, especially if the medium and long-term risks for environmental and public health are considered. Because of their stability and capacity to spread, antibiotic resistant bacteria and resistance genes, abundant in treated wastewater, are probably among the major public health risks associated with water reuse. Nonetheless, these elements are not monitored and are therefore generally overlooked by water management stakeholders. The immense epidemiological hazards of antibiotic resistance demand that these risks be assessed, and possible prevention and/or mitigation measures must be proposed if a sustainable water reuse is intended.

Over the last decades, numerous studies have characterised the evolution and spread of antibiotic resistance in water environments; in particular in wastewater. Although these studies highlighted the relevance of wastewater treatment plants as environmental suppliers of antibiotic resistance, rarely do they offer an opportunity to perform reliable risk assessments. The lack of coherent data obtained under identical or comparable conditions and using the same bioindicators, was considered by the researchers involved the European COST Action DARE (Detecting evolutionary host spots of antibiotic resistance in Europe, TD 0803) as major missing information for a European or even global risk assessment or even for the formulation of corrective measures for a more sustainable wastewater treatment. These are the limitations that motivate the proposed research.

OBJECTIVES

This research aims at developing a database on antibiotic resistance in treated wastewater and in surrounding environments that will serve as a reliable and up-to-date support for future risk assessment studies.

Because, it is presumed that differences in the abundance and diversity of antibiotic resistance genes may occur in distinct municipal wastewater treatment plants

originating from various geographic environments and under different scenarios, a representative set of European/Mediterranean countries (e.g. Austria, Cyprus, France, Germany, Israel, Italy, Poland, Portugal, Spain, UK) will be targeted in the study.

Specific objectives of this proposal include:

- Definition of standardised methods (e.g. sampling, sample processing and analysis)
- Selection of target genetic determinants (e.g. resistance genes, mobile genetic elements)
- Generation of qualitative and quantitative data on each of the selected targeted genetic determinants in wastewater and/or surrounding environments
- Identification of additional genetic determinants (e.g. mobile genetic elements, virulence) using non-targeted metagenomic approaches
- Development of a database, organised by location, sample characteristics and genetic determinants, to be maintained in the future and open to other partners

PROJECT IMPLEMENTATION

Although this activity will be open for researchers interested in participating, it will be promoted by a group of scientists established in the framework of the COST Action TD0803 (DARE). The scientists, equipped with valuable relevant experience, have been collaborating actively for at least 3 years now, and therefore, it is feasible to propose uniform methodologies to be applied by all.

a. Sampling and samples processing

Samples will be collected in each of the participating countries from urban wastewater treatment plant effluents (24 h composite samples) and surrounding environments, and additional data (e.g. type of wastewater treatment, date, temperature, rainfall, sun exposure, BOD, COD, heavy metals) will be concomitantly registered. Samples will be processed within 12 hours after collection. Part of the samples will be cryopreserved for further and future studies, if additional funding (also from other sources) becomes available in the future.

Water samples will be filtered (in triplicate) onto polycarbonate membranes with 0.22 µm porosity and 47 mm diameter. Membranes will be maintained at -80 ºC for further DNA extraction and subsequent analysis (metagenomic, conventional and/or real-time PCR).

b. Targeted sample analysis

Each sample will be characterised using qualitative and/or quantitative methods that assess the occurrence of different antibiotic resistance determinants and mobile genetic elements (AR/MGE). With that objective in mind, it will employ primers specific for a set of clinically important genetic determinants agreed among the partners that are relevant in the wastewater resistome. These genetic determinants, organised in two priority groups (p1 and p2) include: intl1, sul1, sul2, blaCTX-M, blaTEM, qnrS (of group p1), and ermB, aac-6-ib-cr, vanA, mecA, ermF (of group p2).

Determinants included in group p1 will be examined in all samples, whereas those of group p2 will be only assayed in samples collected in sites under the influence of special sources (e.g. animal production, hospital effluents), or when the data for the group p1 justify additional analysis. Because costs may be reduced if a large number of samples is analyzed at once, it is planned that one partner may analyze a single genetic determinant in all samples. In that case, DNA samples must be shipped by courier on dry-ice. For quantitative estimates, the copy number of each of those genes will be normalised to the total rRNA 16S gene present in each sample.

These data will provide a general overview of the prevalence of the different genetic determinants in the distinct regions, offering the possibility of assessing correlations with external factors (type of wastewater treatment, temperature, etc), and inferences about the most reliable indicators to measure the wastewater footprint in terms of antibiotic resistance. Furthermore, in future studies, genetic data of resistances can be analyzed in a phylogenetic or phylogeographic context allowing a possible assessment of similarities between different variants of the same resistance genes originating from different regions.

c. Non-targeted sample analysis

Selected samples will also be examined using Next Generation Sequencing approaches (e.g. Illumina technology, available in some of the participating institutions). This metagenomic data will give further insights into the microbial communities and genetic diversity of the analyzed sites, including presence of pathogens, non-targeted antibiotic resistance genes and mobile genetic elements. Genetic mobile elements (e.g. IS, plasmids, transposons) are known to contribute to horizontal gene transfer (HGT) of antibiotic resistance genes or heavy metal resistance genes in natural aquatic systems, but the relevance of HGT in diverse habitats is not clear and a more in-depth investigation is required. Mobile genetic elements could be identified during metagenome approaches on species or population levels comparing different European wastewater habitats, first. In a subsequent step, molecular biology protocols for qPCR analyses can be developed targeting selected genetic mobile elements as indicators of horizontal gene transfer events in their natural environments. These will be important tools for future risk assessment studies, mainly when water reuse is intended.

d. Effect of stressors on resistance development

It is commonly accepted that wastewater contains numerous micropollutants that may favor the selection of resistant bacteria and/or promote resistance genes transfer. To identify some of these possible effects, transcriptome analyses with selected reference strains, previously isolated from wastewater, will be used to investigate processes of so far unknown induction of gene targets stimulated by the wastewater matrix, which might have an impact on fitness, pathogenicity or genetic exchange. The identification of such new regulator genes is possibly helpful for the development of strategies to minimise the dissemination of antibiotic resistant bacteria, in the future.

Table 1. Project summary for year 1

Activity	Major goals	Nº of samples	Nº of partners involved	Estimated costs in euro	Requested funds in euro
Screening of selected AR/MGE	Establish a pattern of resistance diversity and abundance in wastewater in different European regions	45 (wastewater)	15	100 000	**10 000**
Metagenomic analysis	Assess the overall diversity of AR/MGE, virulence genes, and bacterial groups	10 (wastewater)	5	20 000	**5 000**
				Total:	**15000**

Table 2. Project summary for year 2 (indicative)

Activity	Major goals	Nº of samples	Nº of partners involved	Requested funds in euro
Workshop	Communication of results to stakeholders, scientific community, decision makers)			**ca. 5000**

Phylogenetic analysis of genes	Assess possible paths of dissemination and geographic distribution of resistance determinants transferred by horizontal gene transfer	3-5 genes in all samples examined over year		**ca. 5000**
Transcriptomic analysis	Assess conditions and external factors that may enhance or inhibit the expression of antibiotic resistance genes and/or processes of genetic recombination (horizontal gene transfer; plasmid-chromosome)	10 (bacterial isolates belonging to different taxonomic groups)		**ca. 7 000**
				17000

Notes on suggested activities of year 2015:
Second year of activity:

- Workshop to communicate the results to stakeholders
- Detection of resistance genes, the same detected in wastewater over the first year, among endophytic and leaf bacterial populations in plants (crops or ornamental) subjected to wastewater irrigation;
- Draft of the mobile resistome in wastewater in different Europe regions – lessons to be taken (factors influencing, risks, control measures, etc)
- Selection of some genetic determinants analysed over the first year to proceed for phylogenetic and phylogeographic distribution of genetic determinants, in attempt to assess paths of resistance transmission
- qPCR analysis in metagenome banks obtained during the first year