INNOVATIVE DECENTRALISED AND LOW-COST TREATMENT SYSTEMS FOR OPTIMAL URBAN WASTEWATER MANAGEMENT (IDOUM)

Report to the
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EXECUTIVE SUMMARY

The reuse of treated wastewater is increasingly seen as one of the solutions to tackle the water scarcity problem. Yet, using reclaimed water for non-potable purposes and particularly to irrigate food crops, presents an exposure pathway for antibiotics and Antibiotic Resistant Bacteria and Genes (ARB&Gs) to enter the human food chain. Wastewater reuse is currently of particular concern as a potential source of selective pressure that elevates the levels of antibiotic resistance in native bacteria. These acute strains call for a major shift towards a more localised management of the water cycle, pioneering low-cost wastewater treatment technologies. This project aims at: i) establishing monitoring strategies based on the data-derived prioritization of a set of indicator contaminants and pathogens for domestic wastewater, and ii) developing energy-efficient, cost-effective, and robust treatment systems for the decentralised production of treated wastewater, mainly from domestic wastewater.

In this study, this was achieved through monitoring the antibiotic resistance profiles in passive wastewater treatment using a novel algal consortia. The second component was the assessment of any remediative capacity that passive treatment has on antibiotic resistance. The first goal was addressed by developing and sharing common analytical methodologies for antibiotics and ARB&G among the different participating countries of the Water JPI (Joint Programming Initiative) IDOUM (Innovative Decentralised and low-cost treatment systems for Optimal Urban Wastewater Management) Project (Brazil, France, Germany and South Africa) and by achieving monitoring surveys of domestic wastewater treatment effluents. A diagnostic indicators list has been established including sulfamethoxazole, trimethoprim, ofloxacin and clarithromycin as priority antibiotics across all four countries. The patented passive treatment of wastewater using *Chlorella* algal consortia (reference number 2014/09181 and US20150175457 A1) was assessed in possibly remediating antibiotics either through uptake, transformation, or reduced resistance in comparison to conventional wastewater treatment.

Although the capacity to remediate the antibiotics of concern was not fully validated, there was a clear indication of algal tolerance to the antibiotics and a reduced *E. coli* resistance profiling from the influent to the effluent samples in algal treated wastewater.

CONTENTS

LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
AMR	Antimicrobial resistance
ARB&Gs	Antibiotic Resistant Bacteria and Genes
BOD	Biological Oxygen Demand
COVID-19	Coronovirus Disease 2019
CSIR	Centre for Science and Industrial Research
CuO	Copper Oxide
E. coli	Escherichia coli
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp.
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FLLS	Forward Laser Light Scattering
GDP	Gross Domestic Product
HESI MS/MS	Heated ElectroSpray Ionization coupled to tandem mass spectrometry
HIV	Human Immunodeficiency Virus
HMGU	Helmholtz Zentrum München Deutsches Forschungszentrum für Gesundheit und Umwelt
HPLC	High-performance liquid chromatography
IDOUM	Innovative Decentralised and low cost treatment systems for Optimal Urban wastewater Management
IMC	Isothermal microcalorimetry
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LOD	Limits of Detection
LOQ	Limits of Quantification
MADM	Multiplexed automated digital microscopy
OD	Optical density
PDS	Peroxydisulfate
PL	Project Lead
RSA	Republic of South Africa
RSD	Relative standard deviation
SADC	Southern African Development Community
SCMA	Single-cell morphological analysis
SMR	Scheduled Multiple-Reaction-monitoring
SPE	Solid phase extraction
UHPLC	Ultra-high performance liquid chromatography
UNESP	Universidade Estadual Paulista
USA	United States of America
U.S. FDA	United States (of America) Food and Drug Administration
UV	Ultraviolet
WWTW	Wastewater Treatment Works

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION – THE IDOUM PROJECT

Reuse of treated wastewater is increasingly seen as one of the solutions to tackle the water scarcity problem. Yet, using reclaimed water for non-potable purposes and particularly to irrigate food crops presents an exposure pathway for antibiotics and Antibiotic Resistant Bacteria and Genes (ARB&Gs) to enter the human food chain. Wastewater reuse is currently of particular concern as a potential source of selective pressure that elevates the levels of antibiotic resistance in native bacteria. These acute strains call for a major shift towards a more localised management of the water cycle, pioneering low-cost wastewater treatment technologies.

1.1 ALGAL TREATMENT OF WASTEWATER

The increase in global temperatures, failing water treatment infrastructure as well as the demand for water versus supply in vulnerable countries calls for innovative low-cost solutions. The Council for Scientific and Industrial Research (CSIR) has piloted the treatment of domestic wastewater through algal consortia, using these to decrease nutrients in passive wastewater treatments. **Figure 1** depicts a stepwise progression of the treatment research and its application. The approach is largely enhancing wastewater treatment through introducing dominant algal strains that facilitate nutrient uptake in stabilisation ponds. This is achieved through inoculation of *Chlorella* species, which are known to be pollution tolerant in wastewater (Oberholster et al., 2019). This research has been applied in Limpopo, Mossel Bay and current collaborations are underway in Southern African Development Community (SADC) countries to apply this under different climates.

Figure 1: Progression of algal treatment research application

This project aims at: i) establishing monitoring strategies based on the data-derived prioritisation of a set of indicator contaminants for domestic wastewater, and ii) developing energy-efficient, cost-effective, and robust treatment systems for the decentralised production of treated wastewater, mainly from domestic wastewater. The first goal has been addressed by developing and sharing common analytical methodologies for antibiotics and ARB&Gs among the different participating countries and by achieving monitoring surveys of domestic wastewater treatment effluents over one year.

A diagnostic indicators list has been established including sulfamethoxazole, trimethoprim, ofloxacin and clarithromycin as priority antibiotics while the selection of priority ARB&Gs is still an on-going activity. The second major goal has been addressed by designing wastewater treatment technologies based on the combination of biological-based treatment systems using selected plants and microorganisms (e.g. fungi, endophytic bacteria and microalgae) and the use of low-cost engineered nanostructured materials for catalytically activation of oxidants (persulfate and hydrogen peroxide).

The first half of the project allowed for the isolation of endophytic bacteria from *Phragmites australis*. The possibility of using *Trichoderma spp*. for enhancing the rhizodegradation of antibiotics has also been demonstrated at laboratory-scale using either culture of hairy roots or plants in a phytochamber. The activation mechanisms of Peroxydisulfate (PDS) by nano- and micro-meter Copper Oxide (CuO) particles was investigated in depth, highlighting cupryl ions as predominant reactive species accounting for waterborne antibiotics degradation. Iron oxides and double layered hydroxides were synthetized by including metals like cerium or copper to enhance heterogeneous Fenton processes and were applied to successfully degrade selected antibiotics. All these scientific findings will be collated in future in an attempt to integrate biological (i.e. bio-inoculation of constructed wetlands with endophytic microorganisms) and chemical treatment (i.e. heterogeneous Fenton processes) at pilot-scale. Wetlands inoculated with patented microalgae strains are already running in South Africa while a pilot-scale constructed wetland has been built in France for bioaugmentation experiments and will be implemented with real secondary treated domestic wastewater. The Innovative Decentralised and low cost treatment systems for the Optimal Urban wastewater Management (IDOUM) project aimed to protect freshwater supply, reduce the risk of human exposure to toxic compounds and of antibiotic resistance spreading, and provide access to alternative sources of water (see: http://www.waterjpi.eu/joint-calls/joint-call-2017-ic4water/booklet/idoum-1). The IDOUM project will contribute to the transition from traditional centralised energy-intensive water management practices towards satellite production of treated wastewater for its safe local reuse – avoiding large capital cost and reduced operation and maintenance – supporting local water recycling policies. The technologies could be adopted by small municipalities, farmers' associations or other end-users. Data-derived prioritization and selection of a limited set of antibiotics and ARB&Gs will facilitate data acquisition in the monitoring of wastewater and the assessment of technology efficiency and will contribute towards the development of standards and guidelines for water reuse.

1.2 SOUTH AFRICA'S COMPONENT TO IDOUM PROJECT

The increase in Antimicrobial resistance (AMR) presents a global challenge. According to the recorded use in African countries, consumption of antibiotics in South Africa presents an over- and under use complexity. There is therefore, within the context of increased AMR globally a need to determine the following: are there sufficient feasible methodologies in developing countries to determine the advance in AMR patterns? Could the wastewater treatment plant be a hub for the transfer of AMR and are there low-cost interventions to circumvent this? More specifically, is the use of algal passive treatment additionally effective in the uptake of antibiotics at environmental concentrations? The contribution from South Africa is primarily based on the assessment of antibiotic resistance within the algal passive treatment intervention, which has been effective in reducing nutrients and pathogens in wastewater.

1.3 DELIVERABLES/WORK PLAN FOR IDOUM PROJECT

Table 1: Deliverables of IDOUM

Deliverable name	Lead partner (country)	Date of delivery (dd/mm/yyyy)	Changes, difficulties encountered, and new solutions adopted
WP1	UNESP (Brazil)		
D1.1 List of selected antibiotics and ARB&G key indicators	UNESP (Brazil)	06/02/2020 on-going (as of date of publication)	Difficulties to ship biological samples to Germany for ARB&Gs analysis from RSA and Brazil. A transit through France has been adopted. Monitoring studies for these specific parameters are still on-going.
D1.2 Report on a common protocol to evaluate different technologies in different countries	UNESP (Brazil)	06/02/2020 Completed	None
WP2	HGMU (Germany)		
D2.1 Lab-scale prototype for bio-augmented endophytic bacteria CW and report on experimental tests for key indicators removal	HGMU (Germany)	06/02/2021 Delayed	On-going without any particular difficulties but a delay of 9 months is expected due to the sanitary crisis.
D2.2 Lab-scale prototype for bio-augmented rhizospheric fungi CW and report on experimental tests for key indicators removal	UM-HSM (France)	06/02/2021 Delayed	On-going without any particular difficulties but a delay of 9 months is expected due to the sanitary crisis.
D2.3 Report on experimental tests for key indicators removal by sewage ponds inoculated by selected algae species	CSIR (RSA)	06/02/2021 Delayed	Completed, with an 18 month delay and potential scope for continuation with international project partners.
WP3	UM-HSM		
D3.1 Lab-scale prototype adapted for CuO composite filtration and report on experimental tests for key indicators removal	UM-HSM	06/01/2021 Delayed	Grafting CuO nanoparticles on clay resulted in poor efficiency of the composite. Ceramic pellets will be used as support for CuO or free micrometric CuO pellets. On-going but a delay of 9 months is expected due to the sanitary crisis.
D3.2 Lab-scale fluidized reactor for iron-based heterogeneous Fenton and report on experimental tests for key indicators removal	UNESP (Brazil)	06/01/2021 Delayed	On-going without any particular difficulties but a delay of 6 months is expected due to the sanitary crisis.
WP4	All partners		
D4.1 Report on the quality of the produced treated wastewater with different hybrid technologies at pilot scale	All partners	06/02/2022 Delayed	WWTP was upgraded to a new configuration during winter 2019-2020. A pilot constructed wetland has been built but a delay of 6 months is already anticipated.
D4.2 Report on technology benchmarking in terms of energy consumption and pollutants removal	UM-HSM	06/02/2022 Delayed	

Work was set out according to different work packages, to be tackled by different partners over the duration of the project.

1.4 CURRENT DELIVERABLES – SOUTH AFRICA

Current deliverables for the South African component:

 	D-0 and D-1 Deliverable #1: Inception Report 1) Advance payment with initial report submitted 2) Skype meeting with other country partners 3) CSIR Internal Project team meeting 4) Detailed work plan with planned activities and deliverable deadlines 5) Order consumables to determine antibiotic resistance 6) Initial sampling from wastewater treatment works	March 2019 ✓ ✓ ✓ ✓ ✓ ✓
	D-2 Deliverable #2: Progress Report 1 1) Assess antibiotics needed to determine relevant antibiotic resistance 2) Participate in international project discussions 3) Determine antibiotic resistance in isolated bacteria	Sept 2019 ✓Antibiotics determined ✓International project partners ✓Antibiotic resistance testing
 	D-3 Deliverable #3: Draft Final Report 1) Project evaluation and update with partners 2) Assess algal uptake of four major antibiotics 3) Submission of first IDOUM joint report	March 2021 ✓Second international meeting (Nov 2019 – South Africa) ✓Algal experiments concluded – secondary analysis in Germany ✓Draft joint IDOUM report submitted
 	D-4 Deliverable #4: Final Report	✓March 2022

The next Chapter presents the results from Antimicrobial resistance (AMR) screening.

CHAPTER 2: ANTIMICROBIAL RESISTANCE SCREENING AND PROFILES

2.1 INTRODUCTION

From an economic perspective, there have been earlier research attempts in determining the burden of AMR, looking primarily at patient mortality, morbidity and direct payer as the contributing factors, as well as the loss in Gross Domestic Product (GDP) of a country. Based on a 2012 study, there were variations in the estimations of economic burden, with data from 21 studies. Reviewing this information, it appears the method of statistical analysis can result in the estimation of economic burden from either perspective being unreliable (Naylor et al., 2018). In another recent study, Shrestha et al. (2018) investigated the direct and indirect costs of AMR from a mortality, morbidity, societal as well as the cost per infection, based on *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp. (ESKAPE) microorganisms. This was conducted in the USA (United States of America) as well as Thailand, providing a contrast between developed and developing country scenarios. The costs per full antibiotic course per ESKAPE organism ranged from US$4 to 42 million for the USA and US$3 to 29 million for Thailand. This is based only on the cost of antibiotic treatment, without factors such as social impact costs. This is an indication of the far-reaching economic effects of AMR, in both developing and developed countries.

In developing countries, some of the challenges stem from misuse of antibiotics through unregulated dispensal, patient default in medication and the combined belief in herbal remedies through traditional healing approaches (Ayukekbong et al., 2017). Although based in Cameroon, this study highlighted issues that are relevant to countries where the patient doctor ratio is high and where unskilled personnel handle prescription medication. In South Africa, a 2019 report by Perovic et al. (2018) mapping out the AMR profiles in public hospitals found that the overall resistance had not changed greatly over the years; however an increased resistance of *A. baumanii* was concerning as there are limited treatment options available.

Literature from Humphries and Hindler (2016) indicates the challenge with the increase in antibiotic resistance and the stagnancy in development of new testing methods. This indicates a longer process in detecting emerging resistance, as these processes take days. Moreover, there is a delay in U.S. FDA (United States Food and Drug Administration) updating of the database of breakpoints as well as the emerging antimicrobial treatments being non-FDA approved. The methods used have generally been applied over the past 40 years and there has been a recommendation to move towards other methods in screening that may be more effective as well as the exploration of other antimicrobial agents. The current methods from Syal (2017) are reproduced in Table 2, with a general consensus, however, that plate diffusion disc methods are among the commonly used, in conjunction with minimum inhibitory concentration determination in solid and liquid broth media. This is one of the primary methods applied in the screening of resistance profiles in this chapter.

Table 2: Antimicrobial testing technology

Solid Media Cultures			
Agar Dilution Assay	Bacteria inoculated on agar plates with antibiotic discs of different concentrations	16-24 Hours	Yes
Disk Diffusion	Bacteria inoculated on agar plates with a single antibiotic disk	16-24 Hours	Yes
E-test	Bacteria inoculated on agar plates with a graded antibiotic concentration strips	16-24 Hours	Yes
Liquid Media Cultures			
Broth Dilution Assay	Bacteria inoculated in liquid media with different antibiotics to monitor growth	12-24 Hours	Yes
Automated Instruments			
MicroScan WalkAway	Measure bacterial growth in the presence of antibiotics by recording bacterial turbidity using a photometer	4.5-18 Hours	Yes
Vitek-1/Vitek-2	Measure bacterial growth in the presence of antibiotics by recording bacterial turbidity using a photometer	6-11 Hours	Yes
BD Phoenix	Record bacterial growth in the presence of antibiotics by recording bacterial turbidity and colorimetric changes	9-15 Hours	Yes
Sensititre	Record bacterial growth with antibiotics by measuring fluorescence	18-24 Hours	Yes
Emerging Technologies			
Imaging Based Tools			
Multiplexed automated digital microscopy (MADM)	Image single bacteria growing into colonies with antibiotics and quantify growth rates	3-5 Hours	Yes
Single-cell morphological analysis (SCMA)	Image single bacterial cell's morphology changes on antibiotic action	3-4 Hours	No
oCelloscope	Measure growth of bacterial cells using low resolution optical system	1-4 Hours	No
Non-Imaging Based Tools			
BacterioScan forward laser light scattering (FLLS)	Measures bacterial numbers and sizes on antibiotic action	3-10 Hours	No
LifeScale Microchannel Resonator	Count bacterial cells and morphology changes on single cells post antibiotic action	> 3 Hours	No
Genefluidics	Count 16s RNA increase as a proxy to bacterial growth	4 Hours	No
Smarticles	Bacteriophages which express luciferase on growing cells	-	No
Future Technologies			
Atomic force microscopy (AFM) Cantilever	Measure cantilever fluctuations originating from bacterial motion as a proxy for metabolism	< 2 Hours	No
PIT	Image and Quantify sub-nanometer motion of bacterial cells	< 2 Hours	No
Flow Cytometry	Count viable bacterial cells using dyes	2-3 hours	No
Isothermal microcalorimetry (IMC)	Heat signature of growing cells	3-14 Hours	No

2.2 MATERIALS AND METHODS

Using the commonly applied method of disc diffusion on solid media, the AMR of effluent from Pond 2 to Pond 7 of the Mossel Bay Brandwacht Wastewater Treatment Works (WWTW) described within this chapter has been published as a project output in Genthe et al. (2020).

2.2.1 Site description

The Brandwacht topography in Mossel Bay comprises mountains, hills and valleys that drain westwards into the Brandwacht WWTW River. Land use comprises mainly agricultural activities, residential areas and natural vegetated areas. Mossel Bay's climate is mild throughout the year as the town is situated in the area where the winter rainfall and all-year rainfall regions of the Western Cape Province converge. Mossel Bay receives approximately 333 mm/a rainfall throughout the year. Local weather is influenced by the warm Agulhas current of the Indian Ocean to the south, and by the presence of the Outeniqua Mountains to the North. Rainfall peaks around October, while lowest recorded rainfall in the area occurs during July. Daily maximum midday temperatures range between 18°C in the winter season and 26°C during summer, with the coldest period occurring during winter when temperatures drop during the night (Galley, 2016). Natural land cover in the study area is comprised of Renoster veld and the land use is characterized by cultivated commercial agricultural land (Galley, 2016).

2.2.2 Brandwacht WWTW

Brandwacht WWTW is a waste stabilisation pond system consisting of 7 earth ponds organised in series to treat domestic sewage effluent. Waste stabilisation pond systems are historically designed to allow natural overflow from one pond to another without electricity or other mechanical means. The Brandwacht WWTW is categorised as a micro treatment plant treating less than 0.5 Ml (megalitres) of sewage effluent per day.

It consists of three types of stabilisation ponds, namely (1) anaerobic ponds (Ponds 1A and 1B), (2) a facultative pond (Pond 2), and (3) aerobic (maturation) ponds (Ponds 3-7). All these ponds have different actions and design distinctiveness as summarised below:

<u>Anaerobic Ponds</u>

These ponds operate without the presence of dissolved oxygen with high organic loads. In anaerobic ponds, the Biological Oxygen Demand (BOD) is achieved by sedimentation of solids and subsequent anaerobic digestion in the resulting sludge. A short retention time of one, to one and a half days is commonly used.

<u>Facultative Ponds</u>

In these ponds, aerobic conditions prevail at the water surface and below, while anaerobic conditions prevail in the bottom sediment. Facultative ponds can be differentiated into primary and secondary facultative ponds. Primary facultative ponds receive raw water and secondary facultative ponds receive particle-free wastewater. Facultative ponds are designed for BOD removal on the bases to allow for the development of a healthy algal population, since the oxygen for BOD removal by the pond bacteria is generated primarily via algal photosynthesis. The bottom layer of primary facultative ponds includes sludge deposits that are decomposed by anaerobic bacteria.

Aerobic (Maturation) Ponds

These ponds receive their effluent from the secondary facultative ponds. Maturation ponds show less vertical stratification and are well oxygenated throughout the day. A larger algal diversity can be found in maturation ponds compared to the facultative ponds, with non-motile genera tending to be more widespread. Algae are one of the main driving forces behind treatment within maturation ponds by taking up phosphates, carbon dioxide and nitrogen compounds, while it provides oxygen for heterotrophic bacteria to degrade organic material (Oberholster et al., 2017) (**Figure 2**).

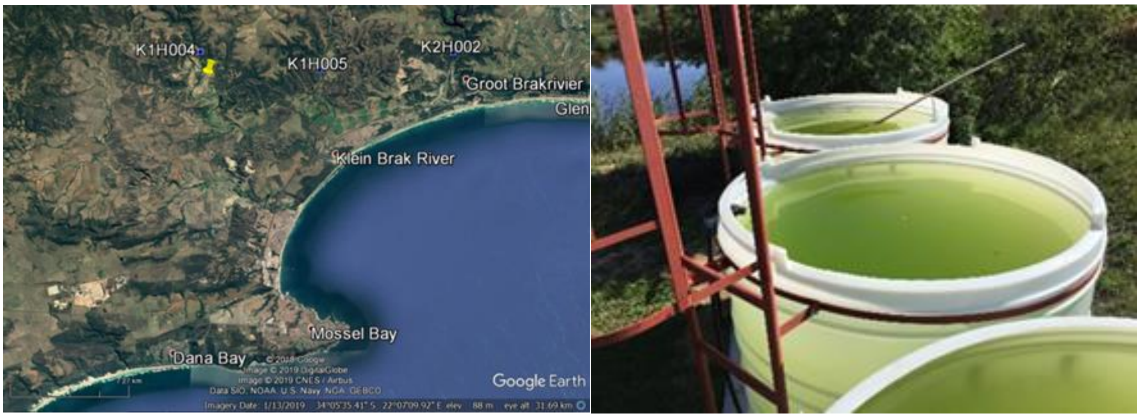

Figure 2: Location of Brandwacht wastewater treatment works in Mossel Bay (left) and algal inoculation tanks (right)

2.2.3 Methodology

E coli analysis and antibiotic resistance assessment method

To determine the changes in AMR and to understand the roles of WWTWs in the development of AMR, samples from Brandwacht WWTW were collected in the Western Cape in South Africa. The antimicrobial susceptibility of effluents and sediments from maturation ponds were determined, using antibiotics used in the control of *E. coli* infections. Sediment samples were collected from maturation pond banks by scooping up sediment at sub-surface levels.

Detection of *E. coli* in samples

Using *E. coli* as an indicator organism, pathogen screening and enumeration was conducted using the Colilert 18 method (IDEXX, South Africa) according to the manufacturer's specifications. Briefly, Colilert nutrient powder capsules were dissolved in 100 mL volumes of surface water samples. For sediment samples, 0.1 g samples of sediment were dissolved in volumes of 100 mL sterile tap water, sealed in 49 well Quanti-Trays and incubated at 35°C, over an 18-hour period. After incubation, samples tray wells were analysed based on colour changes. A change in medium from colourless to yellow indicated the presence of coliforms, whilst fluorescence of the yellow wells under Ultraviolet (UV) light indicated the presence of *Escherichia coli*.

Antimicrobial susceptibility testing

Based on the Colilert 18 method data, 0.1 mL of medium was extracted from fluorescent wells indicating the presence of *E. coli* from the sediment and surface water samples and tested for resistance against various antibiotics. Spread plate antibiotic disc diffusion assays were conducted with the cell suspensions and monitored over a 48-hour incubation period at 35°C on nutrient agar (Merck, South Africa) plates. At least ten samples containing *E. coli* were tested. Overall, thirteen antibiotics were tested, namely, ampicillin, streptomycin, florfenicol, trimethoprim, colistin, enroflaxin, doxycycline, fosfomycin, nalidixic acid, tetramycin, sulphamethoxazole, gentamicin and kanamycin (Oxoid, South Africa). Zones of inhibition around the disc indicated susceptibility to the antibiotic. Zones of inhibition were recorded as either present (susceptible) or absent (resistant), according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) method, with zones of inhibition typically indicated by no growth, when held up about 30 cm from the naked eye.

2.3 RESULTS & DISCUSSION

The majority of *E. coli* isolates were resistant to ampicillin, penicillin, cloxacillin, sulphamethoxazole and trimethoprim (**Table 3, Figure 3**), whereas Streptomycin, Kanamycin, Enrofloxacin, Doxycycline and Gentamycin remained effective against *E. coli* in the majority of isolates tested (**Table 4**) ranging between 70% and 100% sensitivity.

The resistance profile of *E. coli* isolated from sediments differed from those isolated from surface water, with 90% of the surface water isolates being resistant to ampicillin. Isolates from the sediment were less resistant (40%) to ampicillin, whereas nearly all the isolates from the pond and sediment were resistant to sulfamethoxazole. In farm dams only the *E. coli* isolates found in sediment were found to be resistant to fluorquinolone or fluorifenicol.

There is an indication of antibiotic resistance retained within the sediments observed only for Sulphamethoxazole in the Brandwacht wastewater treatment works.

In the wastewater ponds, the number of antibiotics that the *E. coli* were resistant to increased in successive maturation ponds (**Figure 4),** whereas in the *E coli* isolated from sediments, the number of antibiotics they were resistant to started at 9 in pond 1 and only 5 in pond 7.

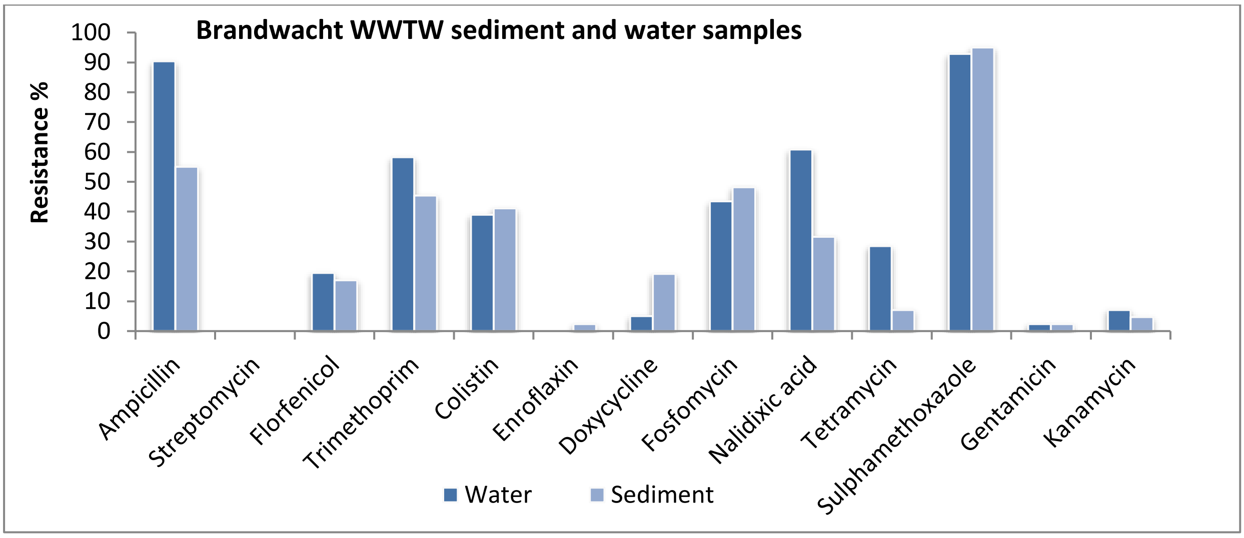

Figure 3: Percentage resistance to different antibiotics from isolated Brandwacht WWTW water and sediment E. coli

Table 3: Antibiotic resistance (if over 50% of isolates were resistant then considered as resistant)

WWTW Water	WWTW Sediment
ampicillin	sulphamethoxazole
nalidixic acid	
sulphamethoxazole	

Table 4: Antibiotic sensitivity (if less than 10% samples were resistant then considered as sensitive)

WWTW Water	WWTW Sediment
doxycycline	doxycycline
enrofloxacin	enrofloxacin
gentamycin	gentamycin
kanamycin	kanamycin
tetracycline	

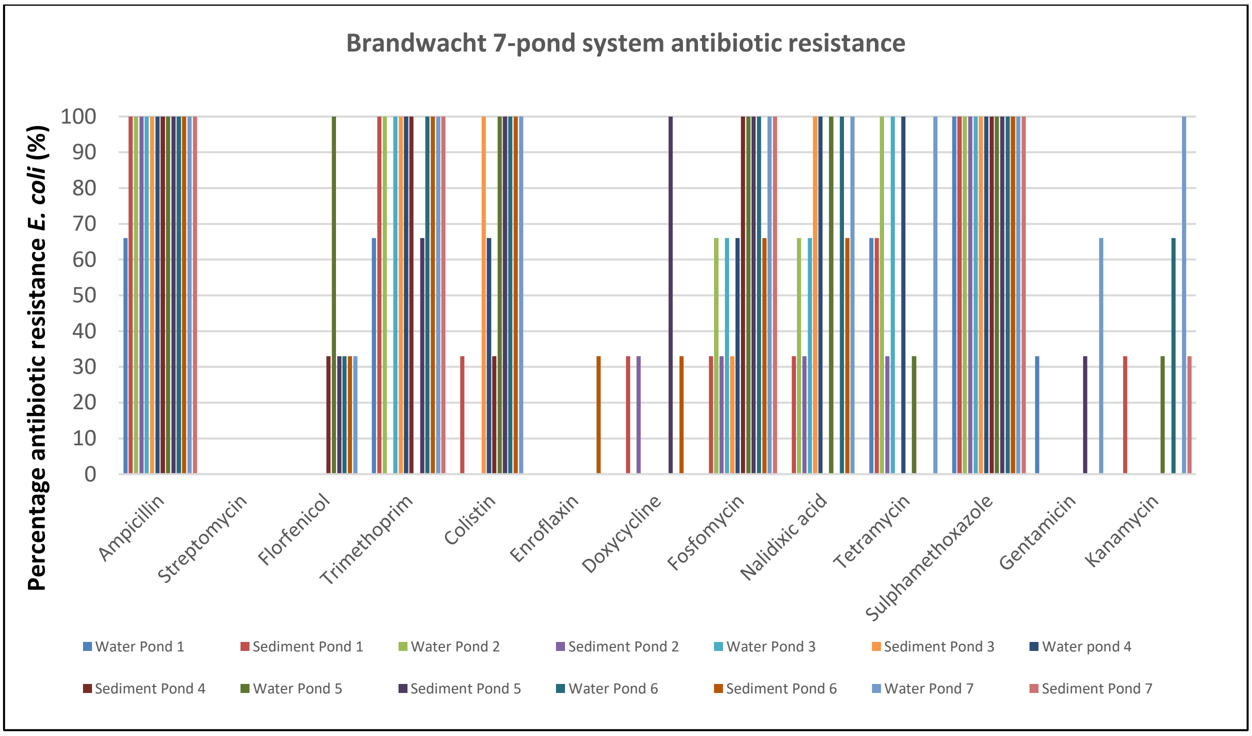

Figure 4: Percentages of antibiotic resistant E. coli isolated from water and sediment samples in the 7 pond Brandwacht WWTW

Sulphonamides such as sulfamethoxazole and/or the sulfamethoxazole-trimethoprim combination have been recorded as the more persistent antibiotics in the environment (Grenni et al., 2019), with an approximate 60 day degradation, with synergistic actions of the metabolite with other antibiotics resulting in a longer degradation time. This has led to the development of resistance genes against this antibiotic. In South Africa HIV (human immunodeficiency virus) positive patients are treated with low doses of this combination as preventative to opportunistic infections (Kaplan et al., 2009); however, 100% of *E. coli* isolates were resistant to sulfamethoxazole, highlighting its relevance in the South African context. This finding is supported by earlier work by Nyamukamba et al. (2019) in the Vaal triangle area, where sulfamethoxazole was detected in higher quantities than other antibiotics tested, which were interestingly below the limit of detection. This is interesting as this is one of the more prevalent antibiotics consumed in the African region in comparison to other global regions.

The advent of passive treatment in developing countries offers a low cost alternative to the conundrum of failing WWTWs which consume large amounts of power and are proving to be a challenge in developed countries as well (Hossain et al., 2010). The comparison of resistance profiles from samples of conventional and this treatment alternative provide useful information on the treatment efficiency and how AMR profiles may change in a passive treatment system. The use of algae to utilise pollutants for nutritional benefit in wastewater, provide an environmentally friendly alternative to the conventional wastewater treatments, with higher retention times (Wang et al., 2010), which may explain the final reduced resistance profile in the sediment of Pond 7 in Brandwacht WWTW, which uses gravitational energy for effluent flow into treatment ponds. In South Africa, a majority of the conventional WWTWs are dysfunctional and not treating water to the required standards (Momba et al., 2006).

Waste stabilisation ponds are a technology used prolifically by South African municipalities due to their simplicity, economy and reliability (Mambo et al., 2014a). In only very limited locations in South Africa are alternative wastewater treatment processes being trialled, such as constructed wetlands (Mthembu et al., 2013) and Algal Integrated Wastewater Pond Systems (AIWPS) or Integrated Algae Pond Systems (IAPS) as a municipal sewage treatment technology (Mambo et al., 2014b). When assessing compliance of this treatment, the effluent produced required additional tertiary treatment to meet the required standards in coliform and total suspended solids (Mambo et al., 2014a). In fact, it is recommended to decision-makers to engage in mitigating risks posed by poorly performing WWTWs by investing in in-stream biotechnologies concurrent to investing in the refurbishment of WWTWs (Mitchell et al., 2014). The comparison of antibiotic-resistance profiles from samples of conventional and this treatment alternative provide useful information on the treatment efficiency and how AMR profiles may change in a passive treatment system. The use of algae to utilise pollutants for nutritional benefit in wastewater provides an environmentally friendly alternative to the conventional wastewater treatments, with higher retention times (Wang et al., 2010), which may explain the final reduced resistance profile in the sediment of pond 7 in Brandwacht WWTW, which uses gravitational energy for effluent flow into treatment ponds.

The use of passive treatment in wastewater treatment, where resistance times are significantly longer than in conventional wastewater treatment systems, may allow the reduction of antibiotic resistance, as shown by the lower numbers of antibiotics that the isolated *E. coli* were resistant to compared with surface water isolates. What is not known is whether the passive systems perform better than conventional WWTWs in reducing the presence of antibiotic-resistant bacteria. In the passive wastewater treatment system, the number of antibiotics that the *E. coli* isolated from the water samples were resistant to, increased in successive maturation ponds, whereas the reverse was seen in the *E. coli* isolated from sediment samples.

These results illustrate the importance of developing a better understanding of antibiotic resistance in wastewater scenarios to ensure remedial measures take place where the greatest benefit can be realised in countries with limited financial resources. Future research is needed to assess the contribution of conventional WWTWs to the growing antibiotic resistance of bacterial isolates and to establish the potential of passive treatment systems. In South Africa (and most developing countries), pathology laboratories and water facility laboratories predominantly make use of the culture method to screen for AMR. This method, among many more sophisticated techniques, remains the gold standard method from a cost perspective. Among the methods to test for antibiotic resistance, there is still no standardised cohesive guide that is widely practised for reporting and comparable analysis in countries with limited resources, thereby making the classical microbiological culture technique the more feasible and standardised approach (Khan et al., 2019). The World Health Organisation (WHO) has implemented an AMR surveillance project which recommends cultivation methods in support of the concept that a simplified, integrated, trans-sectoral surveillance system of bacterial resistance to antibiotics could be implemented on a global basis, the so-called Tricycle project (GLASS 2020). The findings in this study indicate that the theory of wastewater treatment systems being a hub for horizontal antibiotic-resistance acquisition in pathogens is possible based on the increased resistance profiles in the sediment samples of conventional wastewater plants, where increased resistance has been linked to wastewater treatment (Manaia et al., 2018). This is observed in the surface water assessments of all the sites and in the sediment of the passive wastewater treatment plant. The use of passive wastewater treatment to manage persistence of sulphamethoxazole and other persistent antibiotics cannot be concluded within the limits of this research, especially considering that 100% of isolates from each of the seven ponds were resistant to sulphamethoxazole. The sites indicated resistance to the largely used and persistent antibiotics, with increased resistance to fluoroquinolones in the animal influenced samples, this has been reported in other parts of the world where there is intense animal husbandry (Tang et al., 2017; Schulz et al., 2019), which may be due to the unaltered excretion of these antibiotics by the animals, into waters where inadequate treatment exists to effectively degrade or manage the impacts of genetic mutations in exposed bacteria. The comparison of the three sites indicates a global trend in resistance profile influence. Overall, the South African perspective is fairly bleak and complexed with less than 50% of the WWTWs in South Africa meeting national and international water quality standards for wastewater treatment (Mthembu et al., 2013; Mitchell et al., 2014). These findings are proof that South Africa's WWTWs are inadequate to meet the effluent required standards. This has resulted in the urgent need for the development and implementation of innovative systems to resolve the wastewater treatment constraints. This research, among the existing body of the literature, strengthens the call for a more stringent management approach of this challenge, with passive treatment showing a better sensitivity profile. Coupled with the complexities of increased populations, climate change concerns, over- and under-use of antibiotics; the challenge in screening and effective control of AMR in South Africa is critical.

In the next Chapter, the results from the uptake of the selected antibiotics by a consortium of *Chlorella* sp. is presented.

CHAPTER 3: UPTAKE OF ANTIBIOTICS BY *CHLORELLA* SP. CONSORTIA APPLIED IN WWTW

3.1 INTRODUCTION

This chapter presents the uptake of selected antibiotics by the *Chlorella* sp consortium used at the Brandwacht WWTW.

3.2 METHODOLOGY

To assess the uptake of the selected antibiotics, a consortium of *Chlorella* sp. were grown for 4 days at 25°C and then inoculated at 1.2×10^5 cell concentrations into algal media (Sigma-Aldrich) supplemented with additional salts (reference number 2014/09181 and US20150175457 A1) containing 10 or 100 ppb of the following antibiotics: amoxicillin, clarithromycin, sulfamethoxazole, ofloxacin and trimethoprim (Sigma-Aldrich, South Africa). Experiments were conducted in 30 mL volume flasks with abiotic (spiked media) and unexposed algae controls at 7 and 10-day time points on a Labotec benchtop shaker with gentle shaking. Changes in algal response over the exposure period were monitored through optical density (600 nm) and cell counts using the Invitrogen Countess automated cell counter and the Hach DR 3900 benchtop spectrophotometer (Agua-Africa) on day 0, 3, 7 and 10. Chlorophyll (a, b) and carotenoids were extracted from algal biomass using methanol and measured at time 0 and at the end of day 7 and day 10, according to Porra et al., 1989. At the end of each time point (day 7 and 10), 30 mL volumes of media were centrifuged (4000 rpm, 10-15 min), separating the algae from the medium, which was then filtered through 0.45 µm syringe filters (cellulose acetate) prior to antibiotic extraction through solid phase extraction (SPE) cartridges (HLB Oasis, 3 cc, 60 mg), which were pre-conditioned with 6 mL of methanol and then 4 mL of distilled water before passing through the samples. Longer time frames of up to 10 days were set for the experiment, to somewhat mimic the retention times of the passive wastewater treatment ponds.

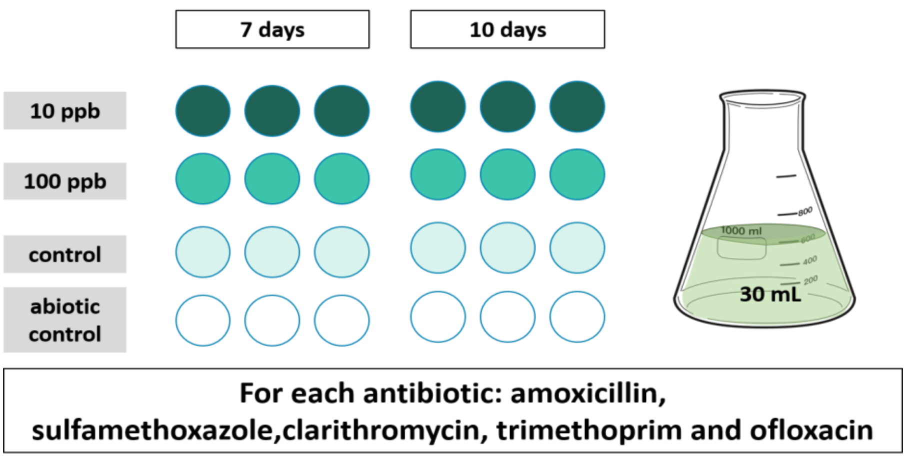

Figure 5: Experimental exposure set up of algal consortia to antibiotics at 10 and 100 ppb concentrations. The High Performance Liquid Chromotography (HPLC) analysis will be performed during the first semester of 2021 at HMGU.

3.2.1 Instrumental and analytical methodology

Briefly, each sample was injected (10 µL) in triplicate into the UHPLC (Ultra-high performance liquid-chromatography) (Dionex UltiMate 3000RS, Gemering, Germany) by an autosampler (Dionex UltiMate 3000TRS, Gemering, Germany) coupled to a triple quadrupole mass spectrometer (HESI-MS/MS, TSQ Quantum Access Max, San Jose, USA), all from Thermo Scientific. The heated electrospray ionization coupled to tandem mass spectrometry (HESI-MS/MS) operated in positive polarity mode with a capillary voltage of 4500 V; nitrogen dumping gas temperature of 350°C; sheath gas pressure 50 AU, auxiliary gas pressure 10 AU, capillary temperature 380°C, skimmer offset of -6, and collision energy together with tube lenses as described in Table 2.

Chromatographic separation was performed on an Accucore PFP column (100 mm x 2.1 mm, 2.6 µm particle size, ref: 17426-102130, Thermo Scientific) with an Accucore PFP pre-column (10 x 2.1 mm, 2.6 µm, ref: 17426-012105) at a flow rate of 0.450 mL/min and a constant temperature of 26°C. The antibiotics were separated using a linear gradient elution made of two mobile phases: 0.1% formic acid in Mili-Q water (A) and 0.1% formic acid in acetonitrile (B). The gradient program was applied as follows: 0-2 min 5% B, 2-8 min 5-100% B, 8-9 min 100% B, 9-9.1 min 100-5% B, 9.1-10 min 5% B. The divert valve was activated from 0-1 min and 8.5-10 min.

Samples were analysed in scheduled multiple-reaction-monitoring (SMR) mode with a scan width of 0.002 m/z and were quantified against a calibration curve with six nominal concentrations ranging from 0.05 to 1.2 mg/L, using three compounds as internal standard and one as a surrogate (both at 0.25 mg/L). The ions selection and the collision energies for quantification purposes were obtained from the auto-selected reaction monitoring.

Table 5: Quantification and diagnostic ions used in LC-MS/MS analyses for algae media samples.

Target compounds	Retention time (min)	Precursor ion (m/z) $[M+H]^+$	Quantifiable product ions (m/z)	Collision energy (eV)	Tube Lens (V)
Amoxicillin	2.53	366.14	114.1*, 134.1*, 208.0*, 86.2	41*, 21*, 32*, 41	80
Ofloxacin	4.93	361.85	318.1, 261.0*, 221.0	19, 27*, 36	100
Sulfamethoxazole	5.07	254.05	108.2, 92.1, 156.2*	19, 23, 16*	103
Trimethoprim	4.58	291.14	230.04*, 123.08, 261.02*	23*, 33, 25*	92
Clarithromycin	7.01	748.48	590.2*, 158.0*, 558.2, 116.0	20*, 27*, 23, 31	95

3.2.2 Validation procedure

The validation procedure followed the ICH harmonized tripartite guideline: validation of analytical procedures (ICH, 2005). Linearity was evaluated using at least three independent calibration curves, each with six nominal standard concentrations (ranging from 0.83 to 20 µg/L) spiked (300 µL) into the 30 mL of algae media. Curves were plotted using the ratio between the standards and the selected IS area. The Limits of Detection (LOD) and quantification (LOQ) were determined with the same curves, using the following formulas: LOD = 3.3 α/S and LOQ = 10 α/S, where α is the standard deviation of the response and S is the average slope of the calibration curves. Recoveries were determined by comparing the area ratio in the spiked matrix with the area ratio of the same concentration in a matrix blank spiked after extraction. Precision was expressed as the relative standard deviation (% RSD) of the replicate measurements, and the accuracy was evaluated as the percentage of agreement between the methods results and the nominal amount of added compound. The experiment was done in South Africa and the media samples were sent to Germany at the end of 2020 for analytical evaluation. Due to the limited access to the LC-MS/MS (Liquid Chromatography with tandem mass spectrometry) equipment during 2021, samples were injected at the beginning of 2022.

3.3 RESULTS & DISCUSSION

The assessment of algal optical density and chlorophyll was conducted as a primary analysis of viability over the 10-day exposure. The optical density shows minimal deviation from the control, with slightly lower turbidity over the 7-day period. This could potentially indicate algal ability to withstand the effects of the antibiotic s amoxicillin and sulfamethoxazole at concentrations of up to 100 ppb (**Figure 6**). A look at chlorophyll *a* production (**Figure 7**) indicates a reduction of chlorophyll *a*, a common method applied to assess algal growth through photosynthetic pigments. Application of the method by Porra et al. (1989) through methanol extraction showed a reduction in the production of this pigment over time. This is an indication of potential algal growth limitation over the exposure period. The measured reductions at the end of day 7 and day 10 are indicated in

Table 6.

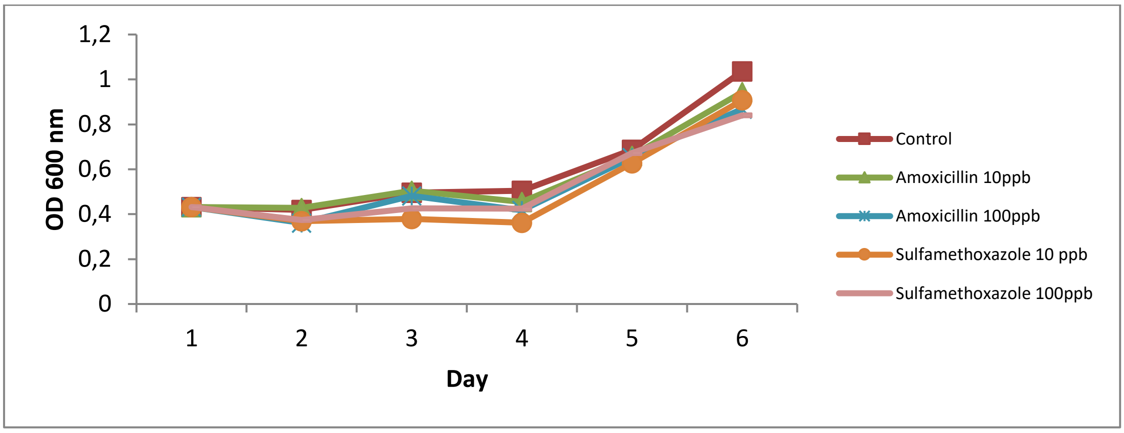

Figure 6: Optical density (OD600) of Chlorella sp. exposed to various concentrations of amoxicillin and sulfamethoxazole over 7 days. The antibiotics showed the most impact on growth on day 2 of exposure, with the growth rate re-established from day 3, with a similar trend as the control. This indicates the potential ability of the consortia to overcome/withstand the effects of the test antibiotics.

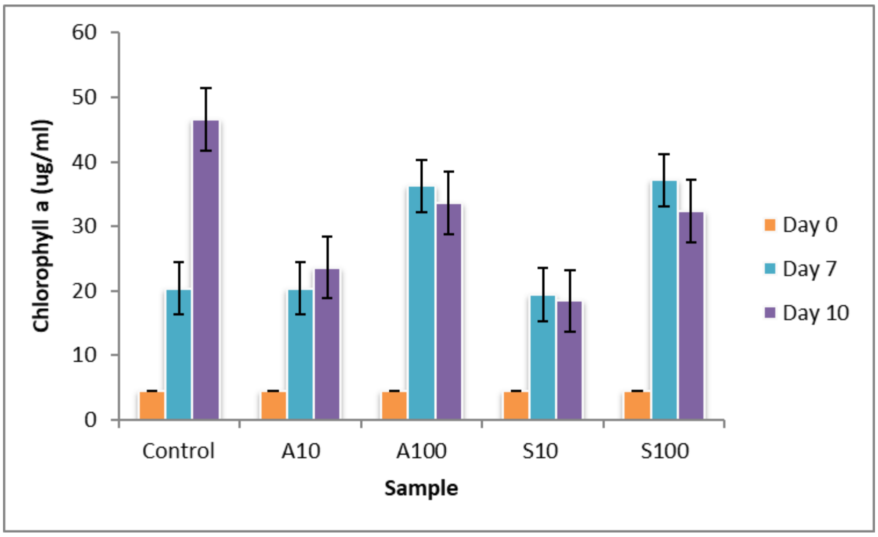

Figure 7: Changes in chlorophyll a of Chlorella sp. consortia exposed to amoxicillin and sulfamethoxazole over a 10-day period, measured at Day 0, 7 and 10.

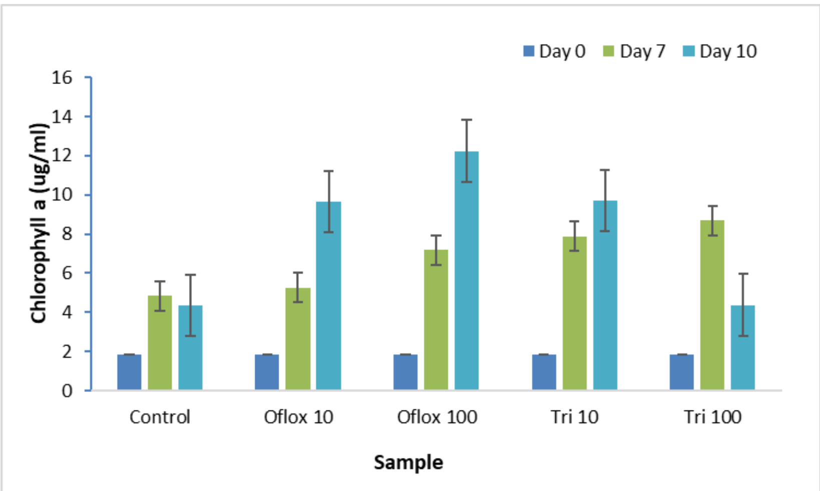

Figure 8: Changes in chlorophyll a of Chlorella sp. consortia exposed to ofloxacin and trimethoprim over a 10-day period, measured at Day 0, 7 and 10.

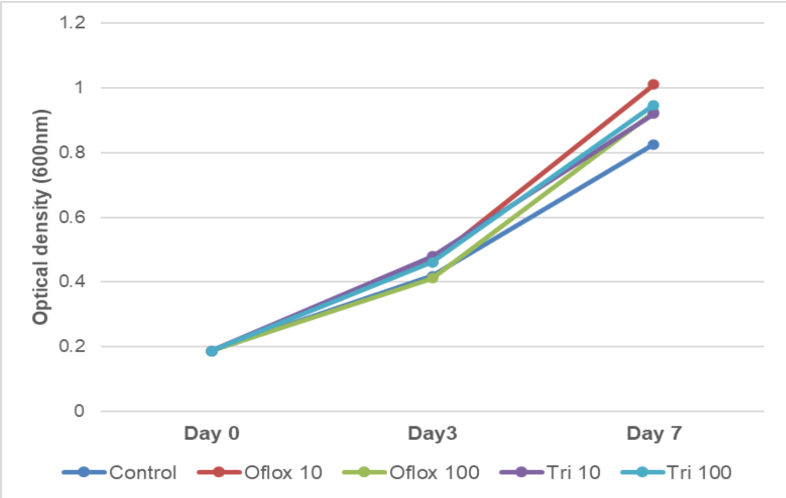

Figure 9: Optical density (OD_{600}) of Chlorella sp. exposed to various concentrations of ofloxacin and trimethoprim over 7 days.

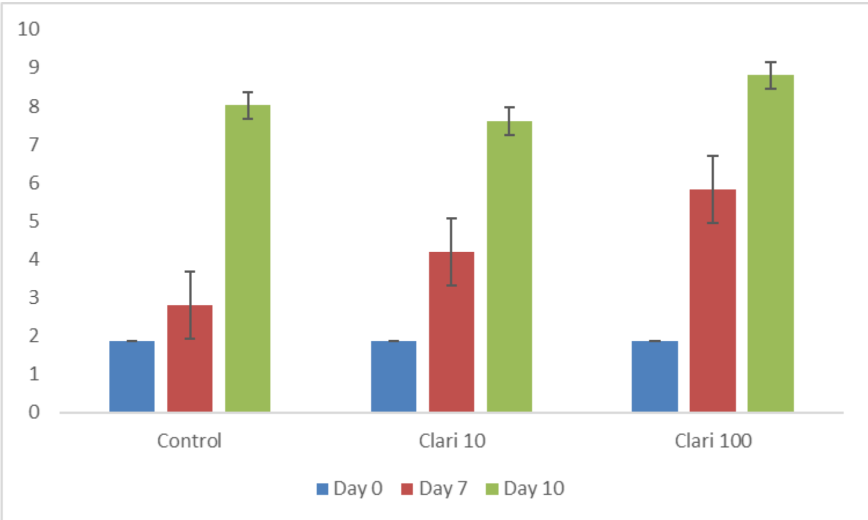

Figure 10: Changes in chlorophyll a of Chlorella sp. consortia exposed to clarithromycin over a 10-day period, measured at Day 0, 7 and 10.

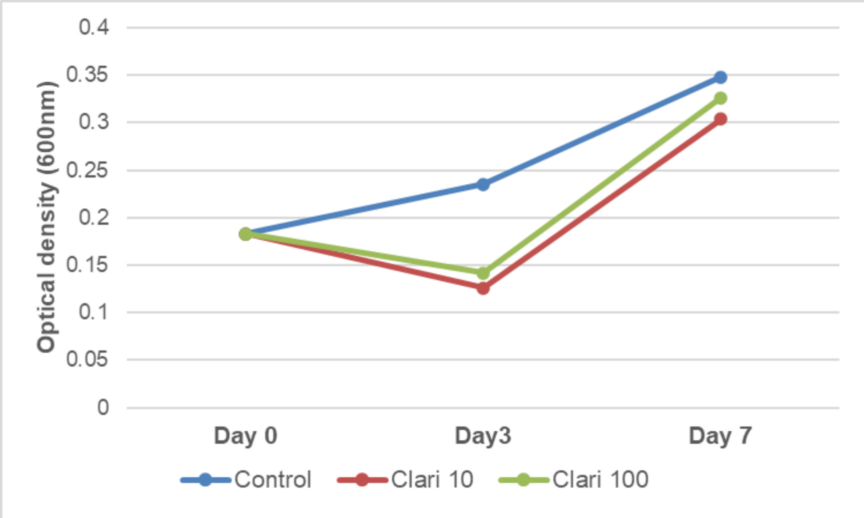

Figure 11: Optical density (OD600) of Chlorella sp. exposed to various concentrations of clarithromycin over 7 days.

Red indicates reduced chlorophyll a production relative to the control samples, whilst green indicates increased chlorophyll production

Table 6: Recorded percentage reductions of chlorophyll a production in algal consortia exposed to amoxicillin and sulfamethoxazole at 10 and 100 ppb concentrations.

Sample	Day 7	Day 10
A10	0.048227	49.27645
A100	77.93836	27.88578
S10	4.603327	60.36422
S100	82.24307	30.62435
Oflox 10	74.1604	121.603
Oflox 100	64.75423	180.819
Tri 10	61.26107	122.858
Tri 100	57.37123	0.47314
Clari 10	79.3943	74.4977
Clari 100	71.46266	102.039

The validated method accomplished all the criteria demonstrating to be valid for the selected antibiotic extraction and quantification. The established range of concentrations were suitable for the experimental design, where RSD% were lower than 20%. Among the selected compounds, amoxicillin demonstrated to be the most unstable, leading to higher LOD and LOQ values. Due to time lapse, only treatment samples with amoxicillin at 10 ppb had detectable concentrations. Concentrations of 8.23 µg/L±1.08 were determined in the treated samples, which might indicate the algae might have some phycoremediation effect, reducing the concentration from 10 µg over 10 days, without a major change in optical density, although chlorophyll *a* was reduced by 49% over this period.

CHAPTER 4: DISCUSSION

One original approach of the IDOUM project emerges from the idea that by combining innovative technologies based on Nature-Based Solutions (NBS) and Advanced Oxidation Process (AOPs) using sustainable catalysts, we can develop cost-effective technological solutions for domestic wastewater treatment tailored to remove emerging contaminants and particularly antibiotic residues and ARB&Gs. This will in turn drive the transition from traditional centralised wastewater management practices towards decentralised practices and will promote safe reclaimed domestic water reuse in irrigation and for other usages at the end. The proposed treatment systems (i.e. bioaugmented constructed wetlands combined with heterogeneous Fenton treatment) has a clear potential to evolve into cost-effective, stand-alone treatment systems that can be easily adopted by rural municipalities and farmers. One of the objectives is to provide a fit-for-purpose water quality scheme not only in compliance with the new European Regulation on minimum quality requirements for water reuse but also at the same time restricting the antibiotic resistance spreading in the environment. This regulation has made wastewater disinfection compulsory. Our project will help policy makers to acquire fundamental understanding of antibiotics and ARB&Gs (antibiotic resistant bacteria and genes) that have the highest environmental and human health risk in wastewater treatment chains.

In observation of the ability to reduce antibiotic resistance, the study shows that passive treatment through algal species is effective in combatting a wider resistance to antibiotics, using *E. coli* as an indicator organism. This brings much needed attention and credibility to the innovations in passive treatment and natural interventions. *Chlorella* has been studied for years as pollution tolerant algal species with antibacterial properties (Hussain et al., 2018).

The findings of the study, although limited by various factors, indicate that there is potential for antibiotic remediation capacity in these algae, although possibly having an impact on the population around the 10-day mark for amoxicillin and sulfamethoxazole (**Table 6**). The other antibiotics have an impact around day 7, with the population recovery noted at 10 days. Without meaningful analytical data on the uptake of these antibiotics, no conclusions can be made on the remediative capacity of the algae, except for the promising potential as observed in amoxicillin concentrations of 10 ppb, where the concentration was reduced by more than 10%. Longer observation times can also assist in accounting for algal population proliferation and display the impacts of these antibiotics over a longer term.

The current analysis from South Africa is preliminary analysis of viability over the exposure period whilst the HPLC analysis was undertaken by the IDOUM partner in Germany (HGMU). Thus far, despite challenges posed by COVID-19, South Africa has completed the majority of independent tasks, save for the additional inputs required by other partners. Delays encountered by IDOUM generally are indicated below.

4.1 CHALLENGES ENCOUNTERED

The IDOUM project started with a delay of 3 months due to some problems in obtaining the signature of the consortium agreement. In addition, Brazilian funding had to start in May 2019 due to the national rules. Consequently, the kick-off meeting could only take place on 24 June 2019.

The original proposal Project Lead (PL) Paul Johan Oberholster (PL for CSIR) left his institution a few months after the beginning of the project. He was replaced by Bettina Genthe who, in turn, retired on 31 December 2020. She was replaced by Luyanda Ndlela as PL.

Mr Andrés Sauvêtre secured a permanent position and left his post-doctoral position at Montpellier University. He was replaced by Dorde Tadic on 1 February 2021.

In 2020 the IDOUM project was heavily impacted by the COVID-19 crisis:

- Montpellier University was closed during April and May 2020 and then reopened but with restricted access of the staff to the research laboratories (e.g. only one person by room). Then, the university was closed for 5 weeks during summertime (August 2020), reopened in September but the access to the research laboratories was again strongly restricted from 1 November to 15 December 2020. In addition, at the beginning of the crisis there was a strong suspicion against the presence of the Coronavirus in domestic wastewaters and the researchers were asked to avoid working with such effluents.

- Helmholtz Zentrum München Deutsches Forschungszentrum für Gesundheit und Umwelt (HMGU) was in lockdown from March to May 2020, reopening thereafter but with restricted access of the staff to the research laboratories (e.g. only one person by room). Then, the centre was again closed for October, reopened for a few weeks but the access to the research laboratories was again strongly restricted from 1 December 2020 until the end of the year; the same restriction continues in 2021 until further notice.

- UNESP was closed in March 2020. Although restricted access allowed to enter research laboratories, large multi-user equipment was not in operation due to lack of technicians because of restrictions due to COVID-19. Restrictions tend to be intensified in February 2021 due to worsening of the sanitary crisis in Brazil.

At CSIR, the implementation of lockdown restrictions affected laboratory access, the procurement and shipment of reagents and therefore challenges and delays in experiments over the period of March to September 2020.

Consequently, WP2 and WP3 had a delay of 9 months and a delay of 6 months was anticipated for WP4. The Consortium agreed to ask for a 6 month extension. This extension was granted and the bigger IDOUM project should end on 5 August 2022.

4.2 MEETINGS HELD

Table 7: Meetings held until time of publication

No	Date	Location	Attending partners	Purpose/ main issues/main decisions?
1	24 June 2019	Paris	P Schröder, B Genthe, R. Pupo, S Chiron	Kick-off meeting Main topics: to get acquainted with one another and to summarize research goals and strategies of the project. Main decisions: · Definition of starting dates of the collaborations · Agreement to encourage publishing of results in open-access format, especially for multidisciplinary articles · Proposal to set up a project website. Finally, we debate about the proposed logos · Organization of the different coming project meetings
2	4-6 Nov. 2019	Mossel Bay	C Cruzeiro, B Genthe, Luyanda Ndlela, R Pupo, A Sauvêtre, S Chiron	1st meeting Main topics: review of ongoing activities, collaborations and planning of upcoming exchange of students. Main decisions: · Presentation of the experimental research for each of the WPs: laboratory and field studies have been initiated; analytical methods are expected to be available shortly, thus allowing to analyse the samples from participating countries. · Pilot-scale treatment unit will be built at Montpellier demonstration site. The possibility to build another one in Munich was discarded. · Decision on students´ visits to partner labs: a PhD student (Nayara De Melo) from UNESP will work at UM-HSM and A. Sauvêtre will work at HGMU. · Selection of a project logo · Agreement on date of next program meeting (Spring 2020 in Brazil).
3	27 May 2020	Remote	C Cruzeiro, B Genthe, P Schröder, Luyanda Ndlela, R Pupo, A Sauvêtre, C Li, S Chiron	2nd meeting · Discussion on how the Covid-19 crisis has impacted the work in different institutions. In the different countries, laboratory facilities have been or are still (Brazil) closed for 2 or 3 months. · WP 2. Cultivation of microorganisms, algae and plants must restart from the beginning after the reopening of the laboratories and probably in Sept. 2020 · WP 3. One Brazilian PhD student has obtained a FAPESP grant to spend 1 year in Montpellier but her venue in France has been postponed to Sept. 2020 because the deliverance of VISA to foreign students has been suspended since by the French Authorities. Chan Li, a PhD student funded by Occitanie Region joined the consortium. She will work on the use of CuO/PDS system for the removal of antibiotics and ARB&Gs from wastewater · Discussion and agreement on the experimental settings of the planned studies

No	Date	Location	Attending partners	Purpose/ main issues/main decisions?
4	17 July 2020	Remote	C Cruzeiro, B Genthe, P Schröder, Luyanda Ndlela, R Pupo, A Sauvêtre, C Li, S Chiron	· 3rd meeting Research activities have been restricted or stopped in all participating countries and there was no possibility to discuss new experimental settings.
5	22 Sept 2020	Remote	C Cruzeiro, B Genthe, Luyanda Ndlela, R Pupo, P Schröder, N de Melo, A Sauvêtre, C Li, S Chiron	4th meeting · Discussion on the possibility to write down an opinion paper on the different approaches adopted in each country/continent for wastewater treatment and reuse. This activity will be led by A. Sauvêtre. · Research activities could restart and there were discussions and agreement on the experimental settings of the planned studies. WP2. Experiments dealing with antibiotic degradation by algae strains selected by RSA partner will be sent to HGMU for analysis. WP3 N. De Melo has started her research activities in Montpellier WP4 Building of pilot-scale constructed wetland has been initiated
6	15 Jan 2021	Remote	C Cruzeiro, B Genthe, R Pupo, P Schröder, N de Melo, A Sauvêtre, C Li, S Chiron	5th meeting Discussion of planned publication as well as draft IDOUM progress report for partners inputs
7	January	Remote	C Cruzeiro, B Genthe, Luyanda Ndlela, R Pupo, P Schröder, N de Melo, A Sauvêtre, C Li, S Chiron	6th meeting Finalisation of draft IDOUM report for submission Planned takeover of projects due to student and PI changes with various partners Discussion of NDA (CSIR and HGMU) for collaboration with partners on patented algal consortium cultivation.
8	April 2021	Remote	C Cruzeiro, B Genthe, Luyanda Ndlela, R Pupo, P Schröder, N de Melo, A Sauvêtre, C Li, S Chiron	Proposal of opinion paper and contributions from different partner countries
9	January 2022	Remote	C Cruzeiro, R Pupo, P Schröder, N de Melo, A Sauvêtre, C Li, S Chiron	Finalisation of opinion paper inputs required progress reporting
10	(upcoming) March 2022	Remote	C Cruzeiro, Luyanda Ndlela, R Pupo, P Schröder, N de Melo, A Sauvêtre, C Li, S Chiron	Presentation of research findings by all partners, inputs into pilot study progress

4.3 PROJECT OUTPUTS – SOUTH AFRICA

The following outputs were achieved at the time of this report publication:

- Website: A website specific to the IDOUM has been created and a project logo has been designed to give more visibility to the project activities to share data inside and outside the consortium and to promote stakeholders' involvement. Protocols, meeting minutes, reports and milestones reached are shared between researchers belonging to the consortium using their account. The files are available for external members only upon request. https://idoum.msem.univ-montp2.fr/en/welcome.php
- Scientific publications: Genthe, B., Ndlela, L., & Madlala, T. (2020). Antimicrobial resistance screening and profiles: a glimpse from the South African perspective. *Journal of Water and Health*, *18*(6), 925-936. Special Edition. https://doi.org/10.2166/wh.2020.034
- Opinion paper on wastewater treatment and reuse approaches in different continents (South America, Africa and Europe). All partners are contributing.

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