



## Research Paper

# Removal of antibiotics, antibiotic-resistant bacteria and their associated genes by graphene-based TiO<sub>2</sub> composite photocatalysts under solar radiation in urban wastewaters



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## ABSTRACT

The present work investigated: (i) the removal of the antibiotics sulfamethoxazole (SMX), erythromycin (ERY) and clarithromycin (CLA); (ii) the inactivation of the total and antibiotic-resistant *E. coli* along with their regrowth potential after treatment; (iii) the removal of the total genomic DNA content; and (iv) the removal of selected antibiotic resistance genes (ARGs), namely *sul1*, *ampC*, *ermB*, *mecA*, as well as species-specific sequences, namely *ecfX* for *Pseudomonas aeruginosa* and enterococci-specific 23S rRNA, by graphene-based TiO<sub>2</sub> composite photocatalysts under solar radiation, in real urban wastewaters. TiO<sub>2</sub>-reduced graphene oxide (TiO<sub>2</sub>-rGO) composite photocatalysts were synthesized by two *ex-situ* synthesis methods, namely hydrothermal (HD) treatment and photocatalytic (PH) treatment, starting from graphene oxide and Aeroxide P25 TiO<sub>2</sub>, and were characterized with various techniques, such as XRD, FT-IR, Raman, XPS, SEM and surface area (BET) analyses. The potential of the synthesized TiO<sub>2</sub>-rGO composites for the removal of the abovementioned antibiotic-related microcontaminants was compared to the efficiency shown by pristine Aeroxide P25 TiO<sub>2</sub> under simulated solar radiation, in real urban wastewater effluents treated by a membrane bioreactor. The results showed that TiO<sub>2</sub>-rGO-PH was more efficient in the photocatalytic degradation of ERY (84 ± 2%) and CLA (86 ± 5%), while degradation of SMX (87 ± 4%) was found to be slightly higher with Aeroxide P25 TiO<sub>2</sub>. It was also demonstrated that more than 180 min of treatment were satisfactory for the complete inactivation and complete absence of post-treatment regrowth of *E. coli* bacteria (< LOD) even 24 h after the end of the treatment, for all examined photocatalytic materials. The least amount of regrowth at all experimental times was observed in the presence of TiO<sub>2</sub>-rGO-HD. Moreover, the synthesized graphene-based photocatalysts successfully removed *ampC* and significantly reduced *ecfX* abundance of *Pseudomonas aeruginosa*, but *sul1*, *ermB* and 23S rRNA for enterococci sequences were found to be persistent throughout treatment with all catalyst types. Finally, the total DNA concentration remained stable throughout the photocatalytic treatment (4.2–4.8 ng μL<sup>-1</sup>), indicating the high total genomic DNA stability in treated wastewater and its resistance to photocatalytic treatment.

## 1. Introduction

As the potential function of treated wastewater reuse as an

alternative source of water supply is now well acknowledged and embedded within international, European, and national strategies, there have been concerted efforts which specifically target a substantial

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increase in recycling and safe wastewater reuse globally by 2030 [1]. Consequently, wastewater treatment plants (WWTPs) offer a highly promising source of recycled water to deal with issues of water scarcity and water quality deterioration in problematic areas. However, the observation of the presence of xenobiotic microcontaminants of emerging concern in treated wastewater effluents makes the task of wastewater reuse a challenging one.

An increase in the abundance of antibiotics due to their overuse, misuse, and incomplete removal by the conventional processes applied in WWTPs, accompanied by an increase in the prevalence of antibiotic-resistant bacteria (ARB) and their associated antibiotic resistance encoding genes (ARGs) has been observed in the last decades in treated wastewater effluents [2,3]. Their presence has been well established in these waters [2,3], suggesting the dominance of these aquatic matrices as potential pathways of human exposure to wildlife strains of ARB, and their associated ARGs [4]. Furthermore, treated wastewater used for irrigation has also been shown to be implicated in the transmission of water-borne pathogenic bacteria, which has led to the implementation of rigorous controls on the discharged effluent microbiological quality intended for irrigation, in various countries [5,6].

ARGs, which may be encoded in bacterial chromosomes or in extrachromosomal plasmids, can elicit biochemical defence mechanisms that allow the survival of bacteria in the presence of the corresponding antibiotic compounds such as in the case of treated wastewater, thus making them increasingly resilient in conditions of environmental stress, *i.e.* during wastewater treatment. Also, plasmid-elicited resistance genes bear the potential of attainment by suitable, previously antibiotic-susceptible bacteria through the horizontal gene transfer (HGT) mechanisms of conjugation, transformation, and transduction [7]. Especially conjugative transfer mechanisms are highly common in natural environments where there is a high density of metabolically active bacterial cells which have a close proximity to each other, making the exchange of genetic material such as ARGs, possible [8,9].

WWTPs, whose operation is typically based on biological treatment, such as the conventional activated sludge process, have been indicated as imperative hubs of ARB development and ARGs dissemination [2,10], as there may be an absence of disinfection process or the existing disinfection process, such as chlorination, ozonation and UV radiation, may be inadequate for the successful inactivation of ARB and removal of genomic DNA and ARGs [11,12]. Consequently, novel disinfection and decontamination processes, such as advanced oxidation processes (AOPs), must be investigated to establish their efficiency for the removal of organic microcontaminants in urban treated effluents. The potential of AOPs for the disinfection of urban wastewater effluents has been previously investigated [13], but the potential of these techniques for the removal of other microbiological components such as ARGs, has not been thoroughly studied. As these processes involve the *in-situ* generation of powerful oxidative transient species, such as free hydroxyl radicals ( $\text{HO}\cdot$ ), they have a strong oxidation potential for a wide range of organic microcontaminants, such as antibiotic compounds, as well as a potential for bacterial cell and DNA damage [14].

Heterogeneous  $\text{TiO}_2$  photocatalysis is an AOP with a wide variety of technological applications, including its use for the inactivation of pathogenic microorganisms [15–18]. It has emerged as a viable option for disinfection, as it possesses the advantage of utilization of natural or artificial solar radiation for its operation, reducing the cost of the process, while making this operation an environmentally sustainable one [15–18]. Due to its associated advantages, various modifications of the  $\text{TiO}_2$  photocatalytic process have been suggested, including coupling of  $\text{TiO}_2$  with graphene-based materials [15,19–24]. Graphene is a two-dimensional nanocarbon material, composed of single-, bi- or few- ( $\leq 10$ ) layers of fused  $\text{sp}^2$  hybridized carbon atoms, forming six-membered rings which are arranged into a honeycomb-like lattice [25–27]. The properties that make it an appealing material for disinfection and decontamination purposes include: (i) high specific surface area, thus increasing adsorption of microcontaminants onto  $\text{TiO}_2$ -graphene

composites; (ii) high transparency due to its one-atom thickness; (iii) extended light absorption range towards the visible region of the electromagnetic spectrum; and (iv) outstanding electron mobility due to its delocalized conjugated  $\pi$  structures. The result of these features is the facilitation of charge transportation and electron/hole separation in  $\text{TiO}_2$ , contributing to the enhancement of the photocatalytic activity of synthesized photocatalytic materials, including  $\text{TiO}_2$ , as demonstrated in various recent studies [15,19–24]. As an outcome, it is suggested that highly conductive rGO could be considered as the ideal material for the improvement of semiconductor photocatalytic materials, as it not only has the potential to serve as an excellent electron acceptor for the restriction of photo-generated electron-hole pair recombination, but also bears various inherent photocatalytic advantages such as its unique two-dimensional carbon structure ( $\text{sp}^2$ -hybridization), its excellent conductivity and a large surface area [15,19–24].

Although numerous papers have been published on the photocatalytic degradation of several organic pollutants in the aqueous phase employing various graphene-based composite  $\text{TiO}_2$  photocatalysts [15,19–24], there is a rather limited number of studies dealing with the photocatalytic disinfection of different microorganisms in the presence of graphene-based  $\text{TiO}_2$  composite materials [14–18,22,28–43]. Indeed, various studies have demonstrated the improvement of disinfection of *E. coli* under natural or simulated solar radiation by graphene-based  $\text{TiO}_2$  composite photocatalysts in different forms, including platelets, nanorod composites, and loose fine powder. Increased antibacterial action was reported by the composites, compared to the efficiency of  $\text{TiO}_2$  alone in the conducted studies. The observed increased inactivation effect compared to the effect of  $\text{TiO}_2$  alone was attributed to the two-dimensional sheet support, to the large surface area and to the increased adsorption capacity [14–18,22,28–43]. For example, Fernández-Ibáñez et al. [14] synthesized  $\text{TiO}_2$ -rGO composites which were compared to Aerioxide P25  $\text{TiO}_2$  in suspension reactors, for the disinfection of water from *E. coli* and *Fusarium solani* spores, under real solar radiation. An increase in the inactivation of *E. coli* was observed using the  $\text{TiO}_2$ -rGO composites compared to  $\text{TiO}_2$  alone, while when the major part of the UVA radiation was cut-off ( $\lambda > 380$  nm) with a methacrylate screen, there was a significant rise in the *E. coli* inactivation time required with  $\text{TiO}_2$ , but no change in the inactivation rate by  $\text{TiO}_2$ -rGO.

Despite the extended investigation of the removal efficiency of synthesized  $\text{TiO}_2$ -rGO of microcontaminants, including antibiotics and bacteria [15–19,29–44], no studies have gone the extra mile to investigate the disinfection potential of the synthesized composites regarding a mixture of various antibiotic-related microcontaminants, including antibiotics, important indicator bacterial species, ARB&ARGs in a real urban wastewater, simulating a real-case scenario, as well as their potential for elimination of bacterial regrowth. Based on the above, the aim of the present study was to synthesize  $\text{TiO}_2$ -rGO composite materials and to investigate for the first time their photocatalytic efficiency in real wastewater, for the abatement of a diverse array of antibiotic-related chemical and microbiological microcontaminants, more specifically: (i) a mixture of various antibiotics including a selection which has been included in the EU Watch list of substances for Union-wide monitoring, as they may pose a significant risk to aquatic environments [44], namely clarithromycin (CLA), erythromycin (ERY), and sulfamethoxazole (SMX); (ii) the inactivation of total bacteria, ARB (*Escherichia coli*) and their regrowth potential after treatment; (iii) the removal of total genomic DNA content which may contain ARGs; and (iv) ARGs (*ampC*, *sul1*, *mecA*, *ermB*) and species specific marker sequences (*Enterococcus* spp. and *Pseudomonas aeruginosa*) using molecular qPCR analyses. Another novelty of this study is the investigation of the above parameters in a real wastewater effluent, which has previously been treated with a Membrane BioReactor (MBR) giving a highly clarified final effluent (final effluent inherent *E. coli* concentrations: 2 CFU 100 mL<sup>-1</sup>, < LOD), thus making it an ideal matrix for the kinetic investigation of the inactivation of spiked indigenous high

bacterial concentrations (approximate initial spiked concentration of  $1 \times 10^6$  CFU mL<sup>-1</sup>) during photocatalytic disinfection experiments. To the authors' knowledge, this is the first study to compare the disinfection, inactivation, and decontamination of an array of antibiotic-related microcontaminants by graphene-based materials in real wastewater effluents, under simulated solar radiation.

## 2. Experimental

### 2.1. Materials and reagents

Clarithromycin (CLA,  $\geq 98\%$ , Chemical Abstract Service (CAS) Number 81103-11-9, molecular formula C<sub>38</sub>H<sub>69</sub>NO<sub>13</sub>, molecular weight 747.95 g mol<sup>-1</sup>), erythromycin (ERY,  $\geq 98\%$ , CAS Number 114-07-8, molecular formula C<sub>37</sub>H<sub>67</sub>NO<sub>13</sub>, molecular weight 733.93 g mol<sup>-1</sup>) and sulfamethoxazole (SMX, CAS Number 723-46-6, molecular formula C<sub>10</sub>H<sub>11</sub>N<sub>3</sub>O<sub>3</sub>S, molecular weight 253.28 g mol<sup>-1</sup>) were purchased from Fluka Analytical and were used as received. Graphite was obtained from Alfa Aesar (graphite powder, synthetic,  $\sim 325$  mesh, 99.9995%) and was used as received. Aeroxide P25 TiO<sub>2</sub> was kindly supplied from Evonik Industries. Ethanol (LC-MS grade) was obtained from Sigma-Aldrich. The solvents used for the chromatographic analysis were acetonitrile (Sigma-Aldrich) and methanol (Fluka). Ultrapure water (resistivity 18.2 M $\Omega$  cm<sup>-1</sup> at 25 °C), employed for solution preparation and as eluent, was prepared on a Merck Millipore water purification system.

### 2.2. Synthesis of TiO<sub>2</sub>-rGO composite photocatalysts

Graphite oxide was synthesized by a modified Hummers and Offeman's oxidation method [45] as described in detail in the Supplementary data (text S1), while exfoliation of graphite oxide to aqueous suspensions of graphene oxide (hereafter denoted as GO) was performed by ultrasonic irradiation, as also described in detail in the Supplementary data (text S1).

The preparation of TiO<sub>2</sub>-rGO composite photocatalysts has been performed by two *ex-situ* methods, namely: (i) by hydrothermal treatment, *i.e.* by heating a suspension of TiO<sub>2</sub> and exfoliated GO in a mixture of ultrapure water and ethanol at elevated temperature (above 100 °C) and pressure (above 1 bar) [46]; and (ii) by photocatalytic treatment, *i.e.* by irradiating a suspension of TiO<sub>2</sub> and exfoliated GO in ethanol under UVA irradiation and anoxic conditions [46,47]. During hydrothermal and photocatalytic treatment, exfoliated GO sheets were partially reduced, thus forming rGO, and at the same time TiO<sub>2</sub> was incorporated in the framework of rGO, thus forming TiO<sub>2</sub>-rGO composites. More details are given in the Supplementary data (text S2).

### 2.3. Characterization of photocatalytic materials

X-ray diffraction (XRD) patterns were collected on a PANanalytical X'pert Pro MPD X-ray diffractometer using Cu K $\alpha$  radiation ( $\lambda = 0.15406$  nm, 45 kV, and 40 mA) in Bragg-Brentano geometry. Fourier transform infrared spectroscopy spectra (FT-IR) of the solid samples were recorded on a Perkin-Elmer Spectrum 1000 spectrometer between 4000 and 400 cm<sup>-1</sup> in KBr pellets. Raman measurements were conducted with a Horiba Lab RAM HR spectrometer equipped with a Peltier-cooled charge coupled device. Excitation was provided by the 514.5 nm line of an Ar<sup>+</sup> laser with a laser power of  $\sim 15$   $\mu$ W focused on the sample by a standard 100 $\times$  objective in a spot with a diameter of  $\sim 1$   $\mu$ m. The signal was collected from an area of 10 $\times$ 10  $\mu$ m<sup>2</sup>, taking advantage of a DuoScan™ scanner, which allows fast scanning of the laser beam on the examined sample. All spectra were normalized to one accumulation time and as to the laser power, to have comparable Raman intensities. Thus, the average power on the sample is very low without reduction in the Raman signal, and the Raman spectrum is more representative of the sample.

Nitrogen adsorption-desorption isotherms were measured at  $-196$  °C on a Quantachrome NOVA 3200e sorption analyzer. Prior to the measurement, all samples were outgassed at 80 °C under vacuum ( $< 10^{-5}$  Torr) for 12 h. The specific surface areas were calculated using the Brunauer-Emmett-Teller (BET) method on the adsorption data in the relative pressure range of 0.05–0.26. The total pore volumes were estimated from the adsorbed amount at the relative pressure of  $P/P_0 = 0.98$ . Scanning electron microscopy (SEM) images were taken with a JEOL JSM-6390LV electron microscope operated at 20 kV. Samples were gently placed on carbon tape, forming flat surfaces, and brought into the instrument chamber for analysis.

The X-ray photoelectron spectroscopy (XPS) measurements were carried out in an ultra-high vacuum system, which consists of a fast entry specimen assembly, a sample preparation, and an analysis chamber. The base pressure in both chambers was  $1 \times 10^{-9}$  mbar. Unmonochromatized AlK $\alpha$  line at 1486.6 eV and an analyzer pass energy of 97 eV, giving a full width at half maximum of 1.7 eV for the Au 4f<sub>7/2</sub> peak, were used in all XPS measurements. The XPS core level spectra were analyzed using a fitting routine, which can decompose each spectrum into individual mixed Gaussian-Lorentzian peaks after a Shirley background subtraction. Regarding the measurement errors, for the XPS core level peaks it was estimated that for a good signal to noise ratio, errors in peak positions were about  $\pm 0.05$  eV. The calibration of the analyzer's kinetic energy scale was done according to ASTM-E 902-88. The samples were in powder form and pressed in stainless steel pellets.

### 2.4. Experimental setup and procedure

To examine the photocatalytic treatment efficiency for the decontamination of antibiotics and inactivation of total bacteria, of ARB and removal of ARGs, a real MBR-treated wastewater effluent was chosen due to its very low inherent antibiotic concentrations (inherent concentrations of the examined antibiotics: 5.5–7.2 ng L<sup>-1</sup>), very low total bacterial concentrations (final effluent inherent *E. coli* concentrations: 2 CFU 100 mL<sup>-1</sup>,  $< \text{LOD}$ ) and high-quality physicochemical characteristics. These characteristics make the MBR-treated effluent an ideal matrix for the kinetic investigation of the removal of the spiked antibiotics ( $C_0 = 100$   $\mu$ g L<sup>-1</sup>) and for the inactivation of indigenous wastewater bacterial concentrations ( $C_0 = 1 \times 10^6$  CFU mL<sup>-1</sup> of *E. coli*) during photocatalytic disinfection experiments.

At the time of the experimental procedure, the MBR pilot unit, which is designed to treat 10 m<sup>3</sup> d<sup>-1</sup> of sewage, was situated at the premises of the University of Cyprus. There are 25 flat-sheet membrane cartridges (Kubota, Japan) with a total functional surface area of 100 m<sup>2</sup> and the membrane nominal pore diameter is 0.4  $\mu$ m. The estimated hydraulic retention time (HRT) was 30 days, while the solids retention time (SRT) was 9 h. More information on the physicochemical characteristics of the water matrix are given in Supplementary data (Text S3).

For the photocatalytic experiments, a laboratory-scale solar simulator Newport type 91193 (1 kW Xenon lamp) was used. The irradiation intensity of the simulator was determined using 2-nitrobenzaldehyde (purchased from Sigma-Aldrich) as a chemical actinometer [48] and it was found to be 63 W m<sup>-2</sup>. Experiments were conducted in a double-walled Pyrex glass batch reactor (inner diameter 8 cm, height 9.5 cm). The experiments were conducted at the inherent solution pH, which was slightly acidic (range of pH 5.2–6.2), under air-equilibrated conditions. Throughout the experimental procedure, the temperature was controlled ( $25 \pm 1$  °C) with the use of a thermostatic bath.

The stock solutions of the examined antibiotics used for the experiments were prepared using the appropriate solvent followed by subsequent dilution in water. In a typical photocatalytic run, an appropriate volume from the stock solution was added to the reactor in order to reach 100  $\mu$ g L<sup>-1</sup> of the substrate in a 300-mL volume of the MBR effluent. After careful bibliographic review, this concentration of

antibiotics has been chosen as it is slightly higher than environmentally-relevant concentrations of the examined antibiotics in the aquatic environment, in order to provide the process efficiency within a measurable time scale and accurately determine the efficiency of degradation of the antibiotics examined in this work [49].

Following, the desired catalyst concentration of  $100 \text{ mg L}^{-1}$  was completely suspended in slurry in the spiked effluent using sonication (10 min) followed by magnetic stirring at 500 rpm (30 min) for antibiotics removal and 60 min for total bacteria and ARB inactivation and genomic DNA, species-specific genes and ARGs removal in the dark, to ensure complete equilibration of adsorption/desorption of the three antibiotics, onto the catalyst surface, or to observe the effect of the catalyst particles on the bacteria in suspension. The catalyst concentration of  $100 \text{ mg L}^{-1}$  was chosen based on a bibliographic review, as it produces a lower operational cost of catalyst use and it gives the advantage of easy catalyst regeneration and removal from the treated suspension. After that period, the solar lamp was turned on for a total photocatalytic treatment time of 60 min for antibiotics, and for 180 min for total bacteria and ARB inactivation and genomic DNA, species-specific genes and ARGs removal examination. The exact procedure for the preparation of the bacterial inoculum is given in the Supplementary data (Text S4). The difference in examined microcontaminant concentration among the times between the first sample in the dark and the next sample at the point when the lamp was turned on and the photocatalytic reaction began ( $T_0$  was considered as the zero photo-reaction time), established the degree of: i) adsorption of the spiked antibiotic compounds, ii) inactivation of the spiked total and ARB, iii) reduction of total genomic DNA or of ARGs due to the presence of the solid catalyst particles, in dark conditions. For the determination of antibiotic concentrations, aliquots (2 mL) were drawn from the photo-irradiated reactor at pre-defined time intervals using 5 mL syringes and were then filtered through  $0.22 \mu\text{m}$  PES filters (Agilent) and injected into 2 mL glass vials for chromatographic analysis.

All experiments were performed in triplicates. Average values are shown as results and the uncertainty that is stated as the standard deviation between measurements never exceeded 15%. Initially, hydrolysis and photolysis experiments were conducted to establish any hydrolysis effect and the impact of sole simulated solar light on the examined microcontaminants.

The determination of the Minimum Inhibitory Concentration (MIC) for the ARB selection was conducted according to the CLSI standard methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically [50]. Detailed information on the MIC determination can be found in Supplementary data (Text S5).

## 2.5. Analytical methods

### 2.5.1. Analytical methods for the determination of physicochemical characteristics

Temperature and pH were measured using a pH meter (EZDO, PL-600). Conductivity was measured using an electronic conductivity meter (EZDO, CTS-506). Dissolved oxygen was measured using an oxygen meter (EZDO, PDO-408). The Chemical oxygen demand (COD) of samples was determined using the Merck Spectroquant<sup>®</sup> cell tests (Merck). The DOC was measured using a TOC analyzer (Aurora 1030).

### 2.5.2. Analytical methods for the determination of total and antibiotic-resistant *E. coli*

The limit of detection (LOD) of the method used for the viability testing (membrane filtration method) of *E. coli* was  $2 \text{ CFU } 100 \text{ mL}^{-1}$ . Detailed information on the methods used for the determination of total and antibiotic-resistant *E. coli* can be found in Supplementary data (Text S6).

In addition to the hydrolytic and photolytic effect investigation on the viability of the selected type of bacteria, a control sample was taken prior to light exposure which was kept in the dark, and plated out twice,

once at the time of sampling and the at the end of the experiment. This control ensured the cell viability without any of the treatments.

As various bacteria were shown to enter a viable but not cultivable (VBNC) state during/after wastewater treatment, they may still be alive but not appearing on cultivation media, while still having the potential of re-activation and regrowth during storage and distribution of treated effluents [51]. Consequently, the photocatalytic experiment duration that was able to completely and permanently halt regrowth of bacteria was investigated. The regrowth of *E. coli* at all experimental times was examined, in order to check whether the reduction in bacteria signified their permanent inactivation, or their injuring and temporary inactivation, which could lead to their regrowth after allowing a specific amount of time for repair/reactivation.

As a result, for the examination of the regrowth of *E. coli* after photocatalytic treatment and subsequent storage in a dark place, 1 mL samples were taken at each experimental time in a sterile container in the dark, and incubated at  $44 \text{ }^\circ\text{C}$  for 24 h. This incubation temperature of the treated samples was selected for a dual purpose: it is the optimum temperature of *E. coli* growth [52] and it represents the real-life scenario of treated wastewater storage in Mediterranean countries, where external tank storage temperatures may be very high, compared to Central and Northern countries. The ratio of the counted colonies ( $C_r$ ) ( $\text{CFU mL}^{-1}$ ) during the regrowth experiment to the colonies counted after photocatalytic treatment ( $C_t$ ) was then calculated, as  $C_r/C_t$ . The ratios calculated that exceeded 1 at any experimental time, were considered as regrowth of damaged/inactivated bacteria that were previously unable to grow on the selective medium. So, bacterial numbers during the regrowth test exceeding the bacterial numbers encountered during the photocatalytic treatment, are considered as repaired/reactivated bacteria that were previously damaged/inactive and were unable to grow on solid media (non-cultivable) due to the photocatalytic treatment.

### 2.5.3. Analytical methods for the determination of total genomic DNA, of species-specific genes and of ARGs

For the determination of the species-specific genes and of ARGs, a separate batch of experiments without any spiked ARB or antibiotics was conducted in order to determine the impact of the photocatalytic process using the examined types of photocatalytic materials, on the inherent DNA concentration and on the prevalence of selected genes. The treated samples were stored at  $4 \text{ }^\circ\text{C}$  in the dark, until analysis, within 24 h of sampling. Due to the low concentration of total genomic DNA in wastewater after MBR treatment, in order to obtain the desired DNA concentration following DNA extraction, 500 mL of the treated solution were used for each DNA extraction.

Primarily, the samples were filtered through 45 mm polycarbonate filter membranes with a  $0.22 \mu\text{m}$  pore size (Millipore). Following, the DNA extraction took place from the collected samples, using the PowerWater<sup>®</sup> DNA isolation Kit (MoBio) following the manufacturer's protocol. The quantity and purity of the DNA extracts was measured using the NanoDrop ND-1000 instrument (PeqLab Biotechnology, Erlangen, Germany).

The DNA content can give an initial indication of the microbial density inside a target matrix, where the transfer and exchange of environmentally- and clinically-relevant ARGs targets among non-pathogenic bacterial fractions can take place via HGT, contributing to the risk of the spread of antibiotic resistance (AR) in the aquatic environment [53,54]. Therefore, the evaluation of the DNA concentration and the calculation of  $\log_{10}$  cell equivalent values (CE) make a reliable means of assessment of the overall relative genetic abundance and of the genetic flow in bacterial communities of water environments.

For the quantitative PCR analyses (qPCR), the specific primers used to quantify ARGs and opportunistic bacteria in 100 ng of total DNA are given in Table 1. A real time quantitative PCR assessment was carried out using the Bio-Rad CFX 96 Touch<sup>™</sup> Real-Time PCR Detection System. The exact analytical procedure followed, is provided in Supplementary

**Table 1**  
The specific primers of qPCR analysis.

Sequence name	Target	Species	Sequence	Reference
<i>ampC-FP</i> <i>ampC-RP</i>	Ampicillin resistance	Beta-lactamase	<i>Enterobacter</i> spp.	[84]
<i>qSUL1_653F</i> <i>qSUL1_719r</i>	Sulfonamide resistance	Dihydropteroate synthase		[85]
<i>ECST784F</i> <i>ENC854R</i>	Enterococcus specific	23S rRNA	<i>Enterococcus</i> spp.	[84]
<i>mecA1FP</i> <i>mecA1RP</i>	Methicillin resistance	Chromosomal penicillin-binding protein 2a	<i>Staphylococcus</i> spp.	[84]
<i>ermB-F</i> <i>ermB-R</i>	Erythromycin resistance	Erythromycin-ribosome-methylase		[54]
<i>EcfXRT-F</i> <i>eefXRT-R</i>	Pseudomonas specific	RNA Polymerase Sigma-70 factor	<i>Pseudomonas aeruginosa</i>	[86]

data (text S7).

### 2.5.4. Analytical methods for determination of antibiotics

The sample extracts were injected directly into the UPLC–MS/MS system. For the determination of inherent antibiotic concentrations inside the MBR-treated effluent, solid phase extraction (SPE) of the samples took place before chromatographic analysis. The SPE procedure was carried out using OASIS HLB cartridges (3 cc, 60 mg) from Waters Corporation (Milford MA, USA). The method of SPE was described previously [55]. Target antibiotics in treated samples were extracted off-line at inherent pH. Prior to the SPE, the samples (100 mL) were filtered through a PVDF membrane filter (0.45 µm, Merck). The cartridges were conditioned with 5 mL methanol and 5 mL ultrapure water. Next, percolation of samples through the cartridges took place by gravity followed by washing with 5 mL water before drying under vacuum for 20 min. Finally, elution took place with 8 mL methanol followed by evaporation under a gentle nitrogen stream to dryness, at 40 °C. The dried samples were finally reconstituted in 1 mL of 25% aqueous solution of methanol.

The presence of antibiotics was monitored on an ACQUITY TQD UPLC–MS/MS system (Waters Corporation) equipped with a triple quadrupole mass spectrometer (TQD, serial number QBA012), operated in positive electrospray (ESI) mode. The most abundant product transition ions used are given in Table 2. The exact analytical protocol is provided in the Supplementary data (text S8).

## 3. Results and discussion

### 3.1. Synthesis of the TiO<sub>2</sub>-rGO composite photocatalysts

As it is well-known from the literature, there are plenty of methods for synthesizing graphene-based composite materials, which can conceptually be divided into two general categories, *i.e.*, *in-situ* and *ex-situ* methods [21]. In the former general synthesis methods (*i.e.*, *in-situ*) an appropriate soluble precursor of the photocatalyst is employed, as well

**Table 2**  
UPLC–MS/MS parameters established for the MRM acquisition mode.

Compound	Precursor ion (m/z) [M + H] <sup>+</sup>	Cone voltage (eV)	Collision energy (eV)	Product ion (m/z) <sup>a</sup>
Clarithromycin	747	40	30	590
	747	40	20	158
Sulfamethoxazole	254	20	16	156
	254	21	25	92
Erythromycin	734.7	35	20	576.4
	734.7	35	30	158.1
	734.7	35	15	116.1

<sup>a</sup> Top: product ion used for quantification; below: the product ions used for confirmation.

as a precursor of graphene (such as GO, amongst others), which are mixed in an appropriate solvent, followed by thermal, chemical, photochemical, or ultrasonic treatment. Moreover, in the *ex-situ* process, the photocatalyst itself, rather than its precursor, is mixed with the graphene precursor in an appropriate solvent, followed by thermal, chemical, photochemical, or ultrasonic treatment, thus resulting in the formation of the graphene-based composite photocatalyst. The main characteristic of the *in-situ* methods is that they usually lead to good interfacial contact between the photocatalyst and the graphene network, while they often suffer from low crystallinity of the photocatalyst. On the other hand, the main advantage of the *ex-situ* methods is that they employ pre-synthesized or commercially available photocatalyst, such as Aeroxide P25 TiO<sub>2</sub>, with well-defined particle characteristics (such as crystallite size, shape, and morphology), which usually remain almost the same during their incorporation in the graphene-based composite photocatalysts [21].

Based on the above, for the synthesis of the TiO<sub>2</sub>-rGO composite photocatalysts, two *ex-situ* synthetic procedures were employed, starting from Aeroxide P25 TiO<sub>2</sub> and exfoliated GO, namely hydrothermal and photocatalytic coupling [46,47], as described in detail in the experimental section and in the Supplementary data. Briefly, in the first preparation method, a suspension of Aeroxide P25 TiO<sub>2</sub> and exfoliated GO in a mixture of ultrapure water and ethanol in a 2:1 volume ratio, was transferred into a Teflon-lined high-pressure reactor and heated at 180 °C for 6 h. During this hydrothermal treatment, exfoliated GO was reduced to rGO, and at the same time, TiO<sub>2</sub> was anchored on the framework of rGO, thus resulting in the formation of the TiO<sub>2</sub>-rGO-HD composite photocatalyst [46]. On the other hand, in the photocatalytic synthetic procedure, a suspension of Aeroxide P25 TiO<sub>2</sub> and exfoliated GO in ethanol was irradiated under UVA radiation in anoxic conditions [47]. Under these experimental conditions, electrons generated by UVA irradiation of TiO<sub>2</sub> were injected into GO, thus reducing the oxygen-containing functional groups and thereby partially restoring the conducting graphene network, while TiO<sub>2</sub> was simultaneously incorporated in the framework of rGO, thus resulting in the formation of the TiO<sub>2</sub>-rGO-PH composite photocatalyst. Moreover, the photo-generated holes from TiO<sub>2</sub> were scavenged by ethanol to form ethoxy radicals, which were further oxidized to CO<sub>2</sub> and water [47].

### 3.2. Materials characterization

XRD patterns of graphite and graphite oxide are shown in Fig. 1a. The diffractogram of graphite shows the sharp 002 diffraction peak at  $2\theta = 26.5^\circ$ , which is characteristic of the highly-organized crystal structure of graphite. On the other hand, the XRD pattern of graphite oxide shows a broad 001 diffraction peak at about  $2\theta = 11.9^\circ$ , which demonstrates the typical loose-layer-like structure of graphite oxide. The interlayer spacing was calculated using Bragg's diffraction law at 0.34 nm and 0.74 nm for graphite and graphite oxide, respectively. The interlayer spacing of graphite oxide is higher than the corresponding

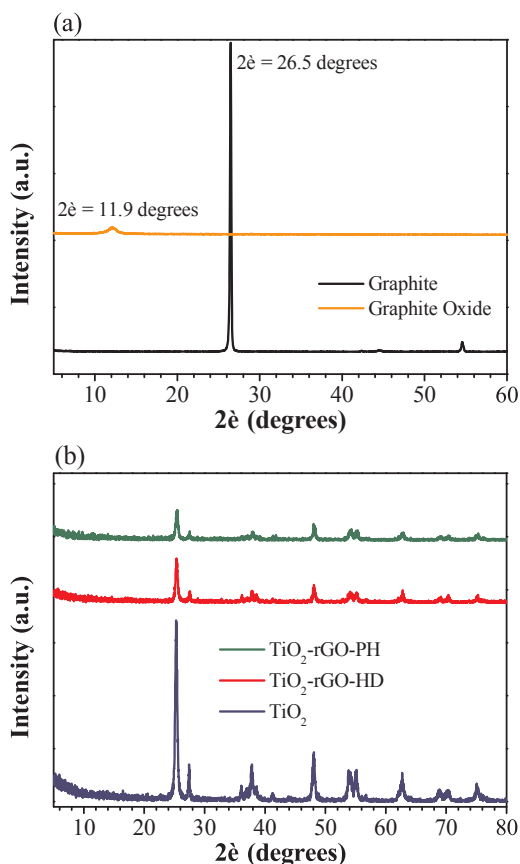


Fig. 1. XRD patterns of: (a) graphite and graphite oxide; and (b)  $\text{TiO}_2$ ,  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH.

value in graphite because of the presence of oxygen-contained functional groups in the oxidized graphene layers of graphite oxide. Fig. 1b shows the diffractograms of  $\text{TiO}_2$ ,  $\text{TiO}_2$ -rGO-HD, and  $\text{TiO}_2$ -rGO-PH. As can be seen in Fig. 1b, the diffractogram of Aerioxide P25  $\text{TiO}_2$  has all the characteristic peaks corresponding to the mixed crystallite phases of anatase and rutile  $\text{TiO}_2$  [56]. Moreover, the diffractograms of the  $\text{TiO}_2$ -rGO composite photocatalysts do not show any significant differences compared to the diffractogram of  $\text{TiO}_2$ , which indicates that the coupling of  $\text{TiO}_2$  with rGO does not cause any significant changes in the crystal structure of  $\text{TiO}_2$ .

Nitrogen adsorption/desorption isotherms were measured using BET analysis to determine the specific surface area of graphite oxide, as well as of  $\text{TiO}_2$  and the synthesized composite photocatalysts. Nitrogen adsorption/desorption isotherms of graphite oxide,  $\text{TiO}_2$ , as well as of  $\text{TiO}_2$ -rGO composites are shown in Fig. S1a and S1b in the Supplementary data, while the calculated BET specific surface areas are shown in Table 3. The BET specific surface area of graphite oxide and  $\text{TiO}_2$  was  $9.182 \text{ m}^2 \text{ g}^{-1}$  and  $48.026 \text{ m}^2 \text{ g}^{-1}$ , respectively, while the BET specific surface area of the synthesized composite photocatalysts  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH was  $48.098 \text{ m}^2 \text{ g}^{-1}$  and  $44.761 \text{ m}^2 \text{ g}^{-1}$ , respectively. The overall shape of the adsorption/desorption isotherms for  $\text{TiO}_2$ -rGO composites indicates an adsorptive behaviour

Table 3  
Calculated BET specific surface areas.

Sample	Specific surface area ( $\text{m}^2 \text{ g}^{-1}$ )
Graphite oxide	9.182
$\text{TiO}_2$ (Aerioxide P25)	48.026
$\text{TiO}_2$ -rGO-HD	48.098
$\text{TiO}_2$ -rGO-pH	44.761

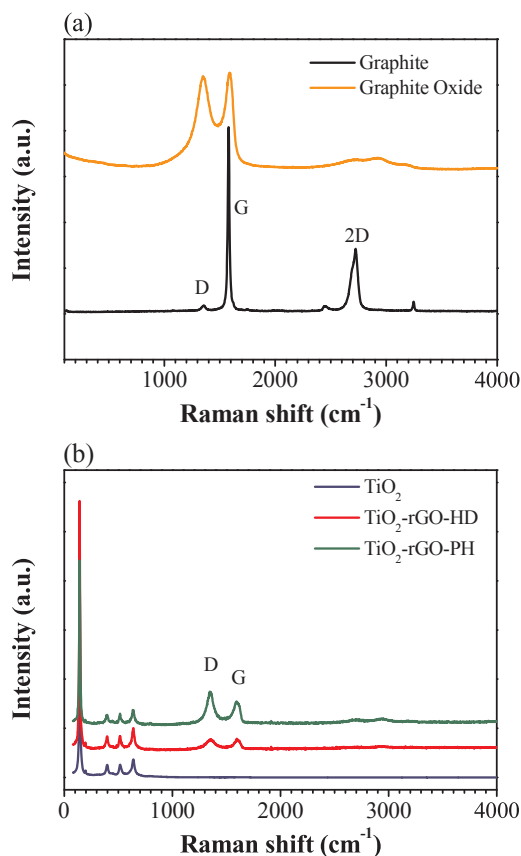


Fig. 2. Raman spectra of: (a) graphite and graphite oxide; and (b)  $\text{TiO}_2$ ,  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH.

characteristic of macroporous materials or materials presenting low porosity.

Fig. 2a shows the Raman spectra of graphite and graphite oxide. As can be seen, the Raman spectrum of graphite is dominated by the characteristic G band at about  $1580 \text{ cm}^{-1}$  and the 2D band at about  $2725 \text{ cm}^{-1}$  [57]. The G band is a common feature of the Raman spectra of all  $\text{sp}^2$  carbon forms and provides information on the in-plane vibration of  $\text{sp}^2$  bonded carbon atoms. The small peak at about  $1350 \text{ cm}^{-1}$  (D band) suggests the presence of  $\text{sp}^3$  defects and its intensity with respect to that of the G band is indicative of the crystalline quality [57], suggesting in our case the high quality of the precursor material. Higher order peaks of smaller intensity are also evident in the spectrum, such as the 2D band, which provides information on the stacking order of graphitic  $\text{sp}^2$  materials. After oxidation, the Raman spectrum is characteristic of oxidized graphitic layers [58]. The Raman peaks of the spectrum of graphite oxide in Fig. 2b are fully consistent with the oxidation of the pristine graphite, featuring significant broadening and small blue shift of the G band peak, strong intensity enhancement of the D band rendering it comparable to the G band, and very broad and weak spectral features covering the 2D band region [58]. The fact that the intensity of the D band is comparable to the intensity of the G band suggests the presence of a high density of defects and structural disorder in the oxidized graphite oxide.

In addition, as can be seen in Fig. 2b, the Raman spectra of the composite photocatalysts  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH contain both the characteristic bands of  $\text{TiO}_2$  in the region  $100 \text{ cm}^{-1}$ – $700 \text{ cm}^{-1}$ , as well as the characteristic bands D and E of graphene-based materials in the region  $1300 \text{ cm}^{-1}$ – $1700 \text{ cm}^{-1}$ . The fact that in the Raman spectra of  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH, the intensity of the D band is comparable to or higher than the intensity of the G band suggests the presence of defects and structural disorders in rGO, caused by  $\text{TiO}_2$  anchored on the framework of rGO. More specifically, the  $I_D/I_G$  ratio of

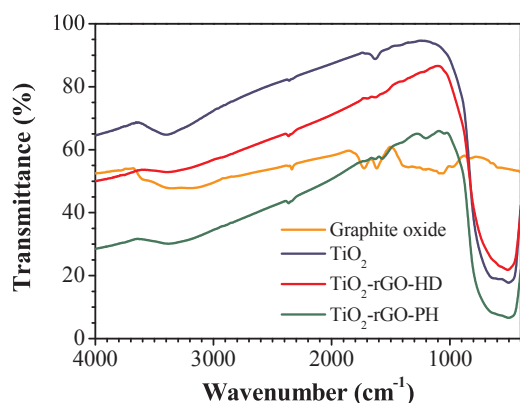


Fig. 3. FT-IR spectra of graphite oxide,  $\text{TiO}_2$ ,  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH.

$\text{TiO}_2$ -rGO-HD was 0.953 while for  $\text{TiO}_2$ -rGO-PH the  $I_D/I_G$  ratio was increased to 1.261. Increasing of the  $I_D/I_G$  ratio is attributed to the formation of more  $\text{sp}^3$  defects in the carbon skeleton of rGO. From the above results, and based on Raman spectroscopy, it can be concluded that the composite photocatalyst  $\text{TiO}_2$ -rGO-HD has lower  $\text{sp}^3$  defects in the carbon skeleton.

FT-IR spectra of graphite oxide,  $\text{TiO}_2$ , as well as of the synthesized composite photocatalysts  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH are shown in Fig. 3. As can be seen, the FT-IR spectrum of graphite oxide contains various characteristic absorption bands which correspond to various functional groups, such as hydroxyl (O–H, broad absorption band from  $3000\text{ cm}^{-1}$  to  $3700\text{ cm}^{-1}$  and centered at about  $3250\text{ cm}^{-1}$ ), carbonyl (C=O, strong absorption band peaked at about  $1725\text{ cm}^{-1}$ ), carbon-carbon double bond (C=C, strong absorption band peaked at about  $1620\text{ cm}^{-1}$ ), and carbon-oxygen single bonds (C–O, absorption bands at about  $1210\text{ cm}^{-1}$  and  $1040\text{ cm}^{-1}$ ). On the other hand, in the FT-IR spectrum of  $\text{TiO}_2$ , the absorption peaks at about  $3410\text{ cm}^{-1}$  and  $1630\text{ cm}^{-1}$  correspond to the OH groups present on the surface of  $\text{TiO}_2$ , and the wide peak at about  $400\text{--}900\text{ cm}^{-1}$  corresponds to the stretching vibration of the Ti–O–Ti bonds. After hydrothermal and photocatalytic treatment respectively, both synthesized composite photocatalysts, i.e.  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH, present a significant decrease in the intensity of all absorption peaks corresponding to oxygen functional groups, a fact that denotes the reduction of graphene oxide under these experimental conditions. In addition, the strong peaks at  $400\text{--}900\text{ cm}^{-1}$  are attributed to the stretching vibration of Ti–O–Ti and possibly to Ti–O–C bonds.

Further characterization of graphite oxide,  $\text{TiO}_2$ , as well as of the synthesized composite photocatalysts  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH was performed by recording their X-ray photoelectron (XP) spectra. The survey scan revealed that the samples contain Ti, C and O as expected. Apart from the wide scan (data are not shown), core level peaks of C1s, Ti2p, and O1s were recorded, as shown in Fig. 4. More specifically, Fig. 4a shows the XP spectra of C1s peaks for graphite oxide,  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH. The binding energy of the main peak of  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH samples is at about  $285.5\text{ eV}$  and is assigned to C–C, and C–H bonds, whereas for graphite oxide the spectra consist of two peaks at about  $284.5\text{ eV}$  and  $286.5\text{ eV}$  assigned to C–C, C–H and C–O, C–OH bonds. In addition, Fig. 4b shows the XP Spectra of Ti2p peak for bare  $\text{TiO}_2$ , as well as for  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH. The binding energy of the Ti2p peak indicates that Ti exists as titanium dioxide on all specimens' surfaces. Moreover, Fig. 4c shows the XP Spectra of O1s for graphite oxide,  $\text{TiO}_2$ ,  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH. The O1s peak of the samples containing  $\text{TiO}_2$  consists of two components; the first one at binding energy  $530.2 \pm 0.1\text{ eV}$  assigned to O in  $\text{TiO}_2$ , and the second one at binding energy at about  $532.0\text{ eV}$  assigned to hydroxyls, carboxyls or adsorbed water. On the other hand, in the graphite oxide sample, the Ti–O component is not present, and the binding energy of the peak is centered at about  $532.0\text{ eV}$ .

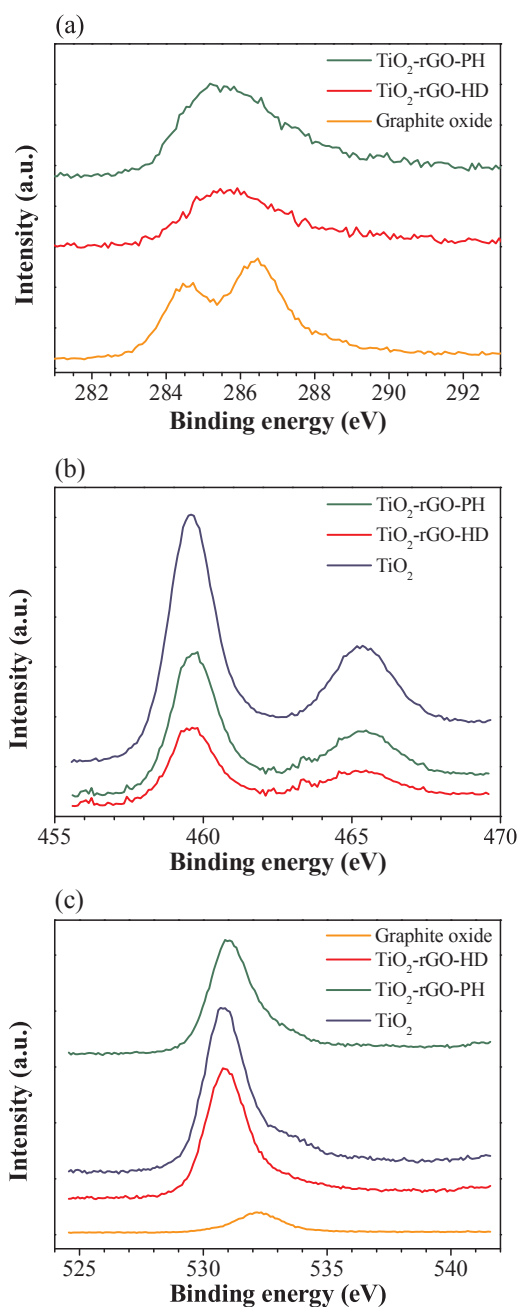


Fig. 4. X-ray photoelectron spectra of the core level peaks of: (a) C1s of graphite oxide,  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH; (b) Ti2p of  $\text{TiO}_2$ ,  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH; and (c) O1s of graphite oxide,  $\text{TiO}_2$ ,  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH.

Scanning Electron Microscopy (SEM) was used to observe the morphologies of graphite oxide,  $\text{TiO}_2$ , as well as of the synthesized  $\text{TiO}_2$ -rGO-HD and  $\text{TiO}_2$ -rGO-PH photocatalysts, and the obtained images are shown in Fig. 5. The morphology of each sample gives a visual understanding of its surface area structure, and of the possible ways to which the surface area may act as a site of adsorption or heterogeneous photocatalysis reactions. As can be seen in Fig. 5, the structure of the surface of each catalytic material is different, and the roughness varies in each material.

### 3.3. Degradation of antibiotics

The concentration of antibiotics in the MBR effluent in the dark (hydrolysis effect) and in the presence of simulated solar radiation (photolytic effect), in the absence of any photocatalytic particles, was

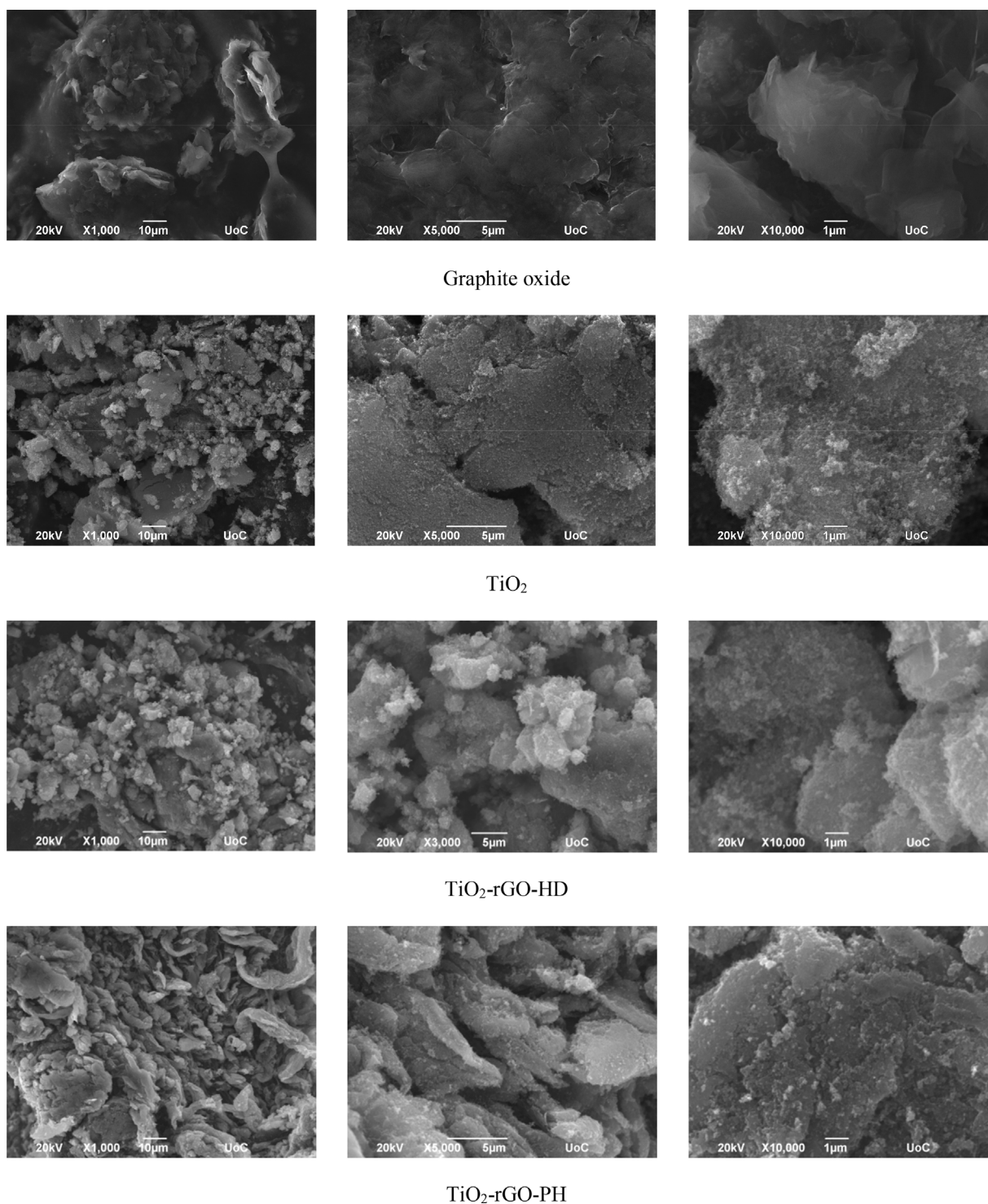


Fig. 5. SEM images of graphite oxide, TiO<sub>2</sub>, TiO<sub>2</sub>-rGO-HD and TiO<sub>2</sub>-rGO-PH. Images on the left side are at the 10 μm scale, while images in the middle and on the right side are at the 5 μm and 1 μm scale, respectively.

initially investigated. The initial hydrolysis and photolysis experiments revealed an average total reduction of the three spiked antibiotics ( $C_0 = 100 \mu\text{g L}^{-1}$ ) below  $10(\pm 5)\%$ .

Following, the potential of TiO<sub>2</sub> to adsorb the three examined antibiotics, in the dark (no catalyst photoactivation) was explored. In more detail, the adsorption of the three antibiotics onto the TiO<sub>2</sub> surface was examined for 30 min in the dark, before the beginning of the photocatalytic process. As can be seen in Table 4, the highest adsorption of CLA was achieved by TiO<sub>2</sub>-rGO-PH, with  $51(\pm 2)\%$  reduction in

Table 4  
Adsorption of the three antibiotics onto TiO<sub>2</sub>, TiO<sub>2</sub>-rGO-HD, and TiO<sub>2</sub>-rGO-PH.

Adsorption and RSD (%)	TiO <sub>2</sub>	TiO <sub>2</sub> -rGO-PH	TiO <sub>2</sub> -rGO-HD
CLA	9 ± 2	51 ± 2	3 ± 2
SMX	7 ± 1	31 ± 4	4 ± 1
ERY	7 ± 1	32 ± 3	20 ± 6



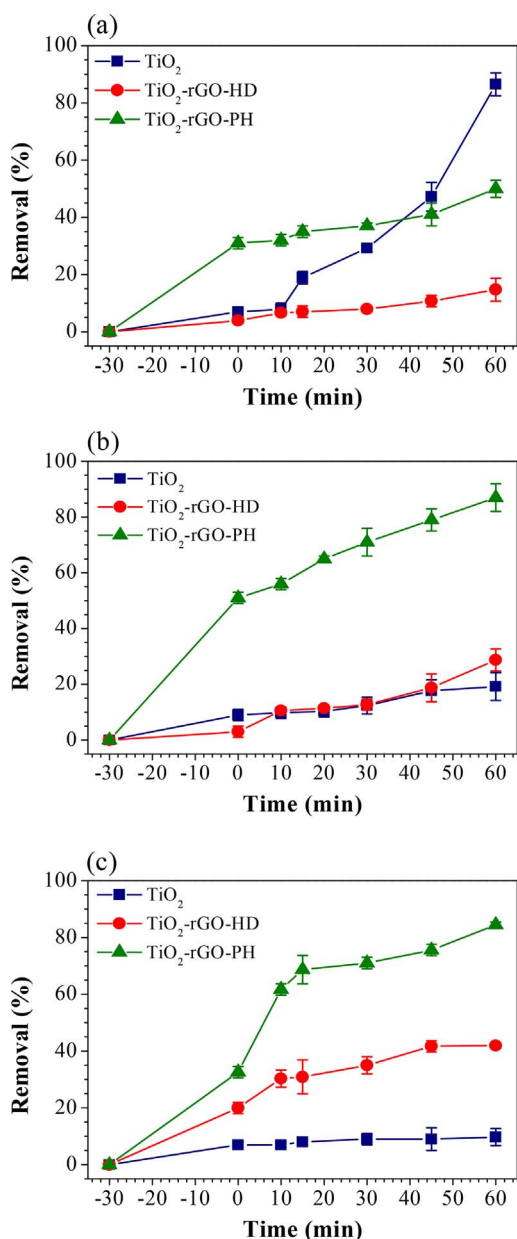


Fig. 6. Photocatalytic removal (%) of the antibiotics (a) SMX, (b) CLA, and (c) ERY over experimental time (min) with the three types of photocatalytic materials used ( $C_{\text{cat}} = 100 \text{ mg L}^{-1}$ ,  $V_{\text{tot}} = 300 \text{ mL}$ ).

concentration before the beginning of the photocatalytic treatment. Moreover, a  $31 (\pm 4)\%$  and  $32 (\pm 3)\%$  decrease were displayed by SMX and ERY respectively, on TiO<sub>2</sub>-rGO-PH. Adsorption of the three antibiotics on TiO<sub>2</sub> and TiO<sub>2</sub>-rGO-HD was lower ( $< 10\%$ ). The only exception to this observation was ERY, which exhibited a decrease in concentration in solution of  $20 (\pm 4)\%$  in the presence of the TiO<sub>2</sub>-rGO-HD composite.

Although the adsorption mechanism of pharmaceuticals onto graphene-based materials has not been completely deciphered yet [59], it has been shown so far that adsorption of organic compounds onto the surface of these materials is attributed to cation- $\pi$  and  $\pi$ - $\pi$  stacking interactions, as well as to electrostatic attractions [59]. This strong adsorption effect is suggested to be dominated by the polarization and electrostatic forces between cations and the permanent quadrupole of the  $\pi$ -electron-rich structures. These reactions are common between amino groups such as between the ones found in the three examined antibiotics, and  $\pi$ -electron-rich structures in TiO<sub>2</sub>-rGO [59].

Next, the effect of photocatalytic treatment on the three antibiotics, spiked in the MBR effluent employing TiO<sub>2</sub>, TiO<sub>2</sub>-rGO-PH and TiO<sub>2</sub>-rGO-HD, was examined. The removal efficiency of the three antibiotics by all studied photocatalytic materials used is shown in Fig. 6a–c. The photocatalytic trials using TiO<sub>2</sub> exhibited a removal of SMX of  $87 (\pm 4)\%$  after 60 min of treatment. These results are in agreement with Hu et al. [60] who reported a complete SMX removal by TiO<sub>2</sub> under UVA irradiation. Moreover, a nearly complete mineralization of SMX was observed by Xekoukoulotakis et al. [61] and Abellán et al. [62] in TiO<sub>2</sub> aqueous suspensions under UVA irradiation.

The removal of ERY by TiO<sub>2</sub> was less efficient with only  $10 (\pm 3)\%$  removal after 60 min of treatment under simulated solar irradiation. Xekoukoulotakis et al. [63] obtained a mineralization of ERY through the examination of the TOC content in an aqueous solution by 90%, while Vignesh et al. [64] also obtained a lower removal of ERY in an aqueous solution by TiO<sub>2</sub>, with a removal of 31.6%.

CLA exhibited a similar photocatalytic degradation response in the presence of TiO<sub>2</sub> to the one shown by ERY, with  $19 (\pm 5)\%$  total removal of the parent compound after 60 min of solar radiation. Overall, CLA is a highly hydrophobic compound with a high acidic stability [65], which may explain its highly recalcitrant character, making its removal through photocatalytic degradation difficult. This persistent behavior of the macrolide antibiotic can be attributed to its rather complex chemical structure. Indeed, CLA contains several functional groups, including various deactivating electron withdrawing groups, which may make electrophilic aromatic substitutions in organic compounds slower and more complicated [66].

The removal of the three examined antibiotics by heterogeneous photocatalysis in the presence of the novel synthesized TiO<sub>2</sub>-rGO composites has been investigated next. As can be seen in Fig. 6, the overall removal of SMX by TiO<sub>2</sub>-rGO-PH reached  $50 (\pm 3)\%$  after 60 min irradiation, which is 37% lower than the corresponding removal obtained by TiO<sub>2</sub>. Moreover, TiO<sub>2</sub>-rGO-PH also achieved a  $86 (\pm 5)\%$  and  $84 (\pm 2)\%$  removal after 60 min irradiation for CLA and ERY, respectively. The TiO<sub>2</sub>-rGO-PH removal potential was found to be higher than the one observed with TiO<sub>2</sub>-rGO-HD, i.e., removal of  $29 (\pm 4)\%$  and  $15 (\pm 4)\%$  for CLA and SMX, respectively, while ERY removal in the presence of TiO<sub>2</sub>-rGO-HD was  $42 (\pm 1)\%$ , which is half the performance of the TiO<sub>2</sub>-rGO-PH photocatalyst.

The observed photocatalytic activity can be attributed to the strong interaction between TiO<sub>2</sub> and GO, which may facilitate the charge transfer from TiO<sub>2</sub> to graphene and hinder electron-hole recombination [19–24]. The electrons may further travel through the  $sp^2$  hybridized network of the graphene towards oxygen groups to generate reactive oxygen species (ROS) which are involved in the chemical oxidation of organic microcontaminants [19–24]. As a result, the provision of open channels and an improved surface area of TiO<sub>2</sub>-rGO composites may play an important role in the increased photocatalytic activity of the synthesized catalysts on the two examined macrolides [19–24]. As observed in Fig. 5, the structure of the catalyst surface after the TiO<sub>2</sub>-rGO-HD and TiO<sub>2</sub>-rGO-PH synthesis method has been altered, providing a transformed surface area on which photocatalytic reactions can take place, in comparison to TiO<sub>2</sub> surface. Overall, the synthesized TiO<sub>2</sub>-rGO materials were shown to exceed the potential of commercially available TiO<sub>2</sub> as to the removal of CLA and ERY, two antibiotics which were proven to be recalcitrant compounds in wastewater treated with conventional biological processes [67,68].

#### 3.4. Determination of inactivation profile of total *E. coli* during photocatalytic treatment

In further experiments, the inactivation of total spiked *E. coli* in the presence of the photocatalysts under simulated solar radiation was studied. Experiments were conducted in the MBR effluent, which was not spiked with any antibiotics, in order to determine the effect of the photocatalytic treatment alone on the survival/inactivation of the total

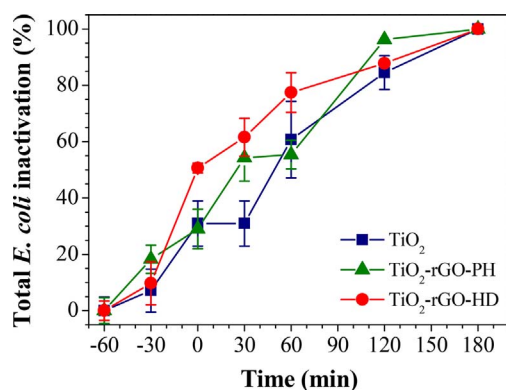


Fig. 7. The inactivation of total *E. coli* (%) by heterogeneous photocatalysis over experimental time (min) with the three examined catalysts, in MBR treated effluent ( $C_{\text{cat}} = 100 \text{ mg L}^{-1}$ ,  $V_{\text{tot}} = 300 \text{ mL}$ ).

spiked bacteria. The aliquot samples were plated on agar that did not contain any antibiotics, to non-selectively grow all *E. coli* and enumerate the total population, including both antibiotic-susceptible and ARB.

Preliminary inactivation experiments in the dark and under simulated radiation for 180 min were carried out in the absence of the catalyst. The results have shown a decrease in the order of less than 1 log of CFU  $\text{mL}^{-1}$  of total bacteria, both in the dark and under simulated solar radiation.

The results of the inactivation of total *E. coli* in the presence of the examined catalysts are shown in Fig. 7. The first 60 min of magnetic stirring in the dark of the MBR effluent inoculated with TiO<sub>2</sub> or TiO<sub>2</sub>-rGO-PH and the bacteria, resulted in an average percentage reduction in the CFU concentration of *E. coli*, of less than 31 ( $\pm 8$ )%. The cause of the reduction may be attributed to the adsorption of catalyst particles onto bacteria, or to the physical damage caused by the direct contact of the catalyst with the bacteria, leading to their inactivation and loss of cultivability. The inactivation of *E. coli* in the presence of these catalysts was less efficient than in the presence of TiO<sub>2</sub>-rGO-HD in the dark, as this material had a more marked impact on the survival of *E. coli*, with a reduction in visible colonies on media of 51 ( $\pm 2$ )%. This difference in efficiency may be attributed to catalyst structure morphology, which may cause increased physical damage to the total *E. coli* population found in the solution. Furthermore, the sensitivity of the indigenous *E. coli* to the presence of catalytic particles even in the dark, indicates their physical vulnerability to cell wall and cell membrane damages, which may consequently lead to rupturing of the cell wall/membrane and allow leakage of cell components to the external environment [69,70].

After illumination with simulated solar radiation of the aqueous slurries containing suspensions of *E. coli* and the photocatalysts, the inactivation of *E. coli* exhibited an increase compared to its inactivation percentage in the dark. After 180 min of radiation, all three photocatalysts achieved a complete absence of cultivable colonies on the growth media ( $< \text{LOD}$ ). The high inactivation after 180 min of treatment indicates the significant impact that the photocatalytic oxidation can have on cellular functions and structures, rendering cells inactive or killing them altogether. It should be noted that the impact of all photocatalysts on total *E. coli* is pronounced both in the dark and under simulated solar radiation. This finding highlights the important contribution of the adsorption of photocatalytic particles on bacteria and of the physical cell damage exerted by the photocatalysts (both processes taking place in the dark and under illumination) to the overall bacterial inactivation observed (29%–51% for all three examined photocatalysts in the dark, followed by 100% inactivation under simulated solar radiation). The pronounced effect of all three photocatalytic materials on bacterial cells further suggests that the damage to cells, both reversible or irreversible, may be leading to loss of growth ability of the damaged cells on the cultivation media used [71].

Foster et al. [72] proposed a sequence of events that may take place in order to inactivate/render non-viable a bacterial cell during photocatalysis with TiO<sub>2</sub>. The authors suggested that the initial contact of bacteria with catalyst particles leading to particle binding onto cells may cause damage to the bacterial cell wall revealing the inner cell membrane. Next, the cell membrane also may get physically injured or rupture, releasing cell contents and allowing catalyst particles to enter the cell and produce further physical damage while hindering vital metabolic and physiological processes inside the cell. At this point, the damage is reversible and may be corrected if the cells are not exposed to other sources of stress. The photocatalytic treatment also produces reactive oxygen species (ROS) such as O<sub>2</sub><sup>•</sup> and HO<sup>•</sup>, which are the main influencing factors in the subsequent inactivation of the damaged cells, because they may confer cell membrane/cell wall modifications, as well as inner cell component damage and modifications [73–76]. Exposed cell component oxidation through the production of ROS, may take place once there is direct cell-catalyst contact [77]. On the other hand, produced ROS have the capacity of cell component damage in close distance from the catalyst particles, thus not only affecting cells in direct contact with catalyst particles but also in close proximity to them [78].

The cellular effect of various graphene-based materials has been demonstrated by Liu et al. [79], where GO and rGO were examined as to their antibacterial activity. The results of the study showed a higher loss of *E. coli* viability by GO than by rGO, with the biggest fraction of cell inactivation occurring in the first hour of treatment. In another study by Fernández-Ibáñez et al. [14], TiO<sub>2</sub>-rGO composites synthesized using the photocatalytic reduction of exfoliated GO by TiO<sub>2</sub> under UV radiation, were used to disinfect water contaminated with *E. coli* and *Fusarium solani* spores under real sunlight. Moreover, the production of singlet oxygen was measured using fluorescence intensity of a singlet oxygen specific fluorophore, relative to a control sample of distilled water. An enhancement in the rate of *E. coli* inactivation was observed compared to TiO<sub>2</sub>, while the rate of inactivation of the *Fusarium solani* spores remained the same for both catalysts examined. In another study by the same authors [29], it was found using probes, that the main ROS involved in the TiO<sub>2</sub>-rGO photocatalytic disinfection of *E. coli*, were determined to be hydrogen peroxide, HO<sup>•</sup> and O<sub>2</sub><sup>•</sup> under UV-vis radiation, while only O<sub>2</sub><sup>•</sup> was found under visible radiation.

### 3.5. Development of the prevalence profile of antibiotic-resistant *E. coli* during photocatalytic treatment

The developed profile of antibiotic-resistant *E. coli* during photocatalytic treatment (Fig. 8a–c) have been analysed, in order to observe potential trends in photocatalytic inactivation, that may be associated to the resistance of the bacteria to a specific antibiotic.

The most pronounced effect of the catalyst particles alone on *E. coli*, after 60 min of bacterial exposure in the dark, was shown in the case of TiO<sub>2</sub> (i.e. 75 ( $\pm 7$ )% reduction in ERY-resistant *E. coli* and 39 ( $\pm 6$ )% reduction in SMX-resistant *E. coli*). This finding indicates the increased adsorption effect or susceptibility of antibiotic-resistant *E. coli* to cellular damage and loss of cultivability in the presence of the photocatalysts, due to physical damage occurring on the cell surface due to contact with photocatalyst particles.

Additionally, it was seen in this study that the cultivability of ERY- and SMX-resistant bacteria was lost in the dark, even before the photocatalysis began. Sub-lethal concentrations of a stressor, such as the concentration of the antibiotic, in combination to the exposure to suspended catalyst particles, may have produced added environmental stress that may lead to the induction of a VBNC state in the bacteria [51]. The subsequent repair and re-activation of bacteria therefore is an important parameter to be investigated when dealing with processes that may put bacteria in such a VBNC state, to ensure that the damage caused is leading to permanent inactivation, and not to the post-treatment re-growth of bacteria.

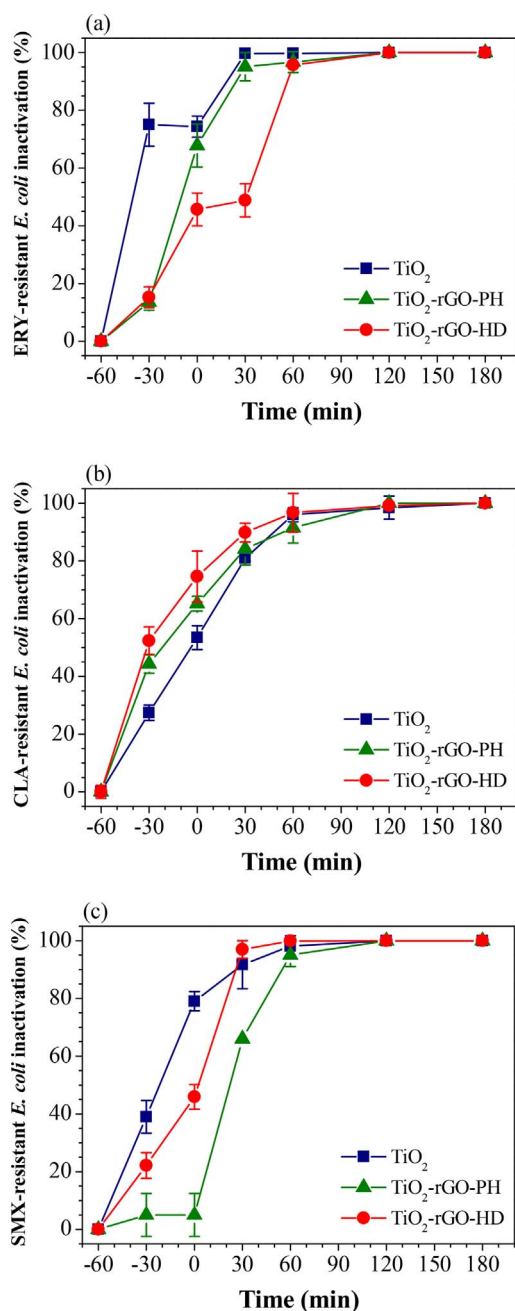


Fig. 8. The inactivation (%) of antibiotic-resistant *E. coli* throughout the photocatalytic treatment (min) for (a) ERY-resistant *E. coli*, (b) CLA-resistant *E. coli* and (c) SMX-resistant *E. coli* ( $C_{\text{cat}} = 100 \text{ mg L}^{-1}$ ,  $V_{\text{tot}} = 300 \text{ mL}$ ).

As the photocatalytic treatment began and the photocatalytic oxidation reactions were initiated, the most rapid inactivation of antibiotic-resistant *E. coli* was observed in the presence of ERY, as  $\text{TiO}_2$  achieved its complete inactivation after 30 min of treatment. ERY-resistant *E. coli* bacteria were not visible on the selective media after 120 min of treatment in the presence of all photocatalysts while the inactivation of CLA- and SMX-resistant *E. coli* followed a similar pattern. It is suggested from this finding that the efficient inactivation of antibiotic-resistant *E. coli* to all examined antibiotics, shows that AR mechanisms do not also provide cellular protection in the presence of photocatalytically produced ROS, or protection mechanisms to resist physical damage.

Overall, there was a high variation in the development of the AR profiles and in the post-treatment regrowth potential of both examined types of bacteria, which changed according to the combination of

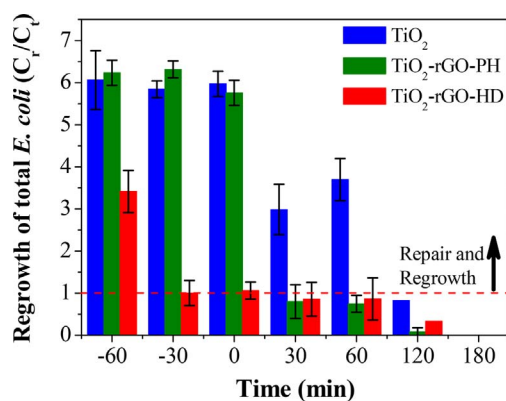
treatment-antibiotic present. This observation may also indicate variation in the genetic profiles of the autochthonous community of *E. coli* that was isolated from real wastewater, and was used for the photocatalytic experiments. The variability in AR profiles of the bacteria used also reflects the real-life scenario, as a large range of phenotypic as well as genotypic profiles exists in real wastewater matrices.

There is limited research regarding the effect of heterogeneous  $\text{TiO}_2$  photocatalysis, and especially of newly synthesized photocatalytic materials such as graphene and its derivative composites such as  $\text{TiO}_2$ -rGO, to support and explain the above findings. The research conducted so far, regards a limited number of bacterial species. According to Tsai et al. [77], altered susceptibility to  $\text{TiO}_2$  photocatalysis may come about through the mutation of genes involved in the ROS resistance. In addition to cellular mechanisms to resist ROS damage, ARB may present altered resistance to photocatalytic treatment through gene regulation and stepwise gene mutation acquisition, such as plasmid-mediated HGT. This process may equip bacteria with genetic material, which can combine resistance to photocatalytic treatment and to antibiotics [80]. Moreover, the reduced antibiotic susceptibility of Gram-negative bacteria which include *E. coli*, compared to antibiotic-positive bacteria has also been previously attributed to outer cellular component alteration/destruction. More specifically, these components involve cell wall structures which include additional structural barriers such as lipopolysaccharides to the entry of inhibitory or harmful substances, as well as to their ability to produce catalase enzymes, which limit the formation of ROS during photocatalytic treatment [81].

### 3.6. Regrowth potential of *E. coli* after the end of the photocatalytic treatment

Even though the ability of bacteria to grow in cultivation media may be lost after photocatalytic treatment, the potential of their repair and regrowth, given the necessary recuperation time, should not be neglected, as this phenomenon was shown to take place in various studies examining the disinfection potential of various heterogeneous photocatalysts [15,82]. Therefore, the regrowth potential of *E. coli* at different experimental times was studied thereafter during photocatalysis in order to establish the experimental time which is capable of permanently inactivating *E. coli*, thus avoiding the re-activation and regrowth of the bacteria during storage of the treated effluent. All photocatalysis experimental times were examined in this study, in order to observe their inactivating capacity (counts of bacterial colonies immediately after treatment), compared to the regrowth potential generated at each experimental time (counts of bacterial colonies after 24 h of storage). Samples for regrowth examination were incubated at  $37^\circ\text{C}$  for 24 h in the dark, and then plated on TSA agar. The ratio of the enumerated colonies ( $\text{CFU mL}^{-1}$ ) in the incubated samples after 24 h of storage ( $C_r$ ) to the colonies enumerated in the obtained samples at the time of treatment ( $C_t$ ) was calculated, and expressed as  $C_r/C_t$ .

Fig. 9 shows the findings of the regrowth study done on *E. coli* after photocatalytic treatment, at different experimental times. The results have shown a considerable regrowth of *E. coli* during the dark treatment, in the presence of the photocatalytic particles, despite the original reduction in colony counts due to physical cell damage. More specifically, a  $C_r/C_t$  ratio of 6 was found for Aeroxide P25  $\text{TiO}_2$  and  $\text{TiO}_2$ -rGO-PH, signifying the growth on solid media, of higher counts of *E. coli* by 6 orders of magnitude after 24 h of storage, than the ones found in the plated samples just after treatment. The same phenomenon was observed until the time that the solar simulator was turned on, and the photocatalytic process began. In contrast, treatment with  $\text{TiO}_2$ -rGO-HD, achieved a reduced bacterial regrowth during the first 60 min of dark treatment compared to the other two photocatalysts ( $C_r/C_t$  ratio of 3). This finding may suggest that the physical contact and bacterial surface damage incurred by  $\text{TiO}_2$ -rGO-HD may produce a more severe and permanent inactivation effect on bacteria, thus reducing the counts of *E. coli* that are thereafter cultivable on solid media.

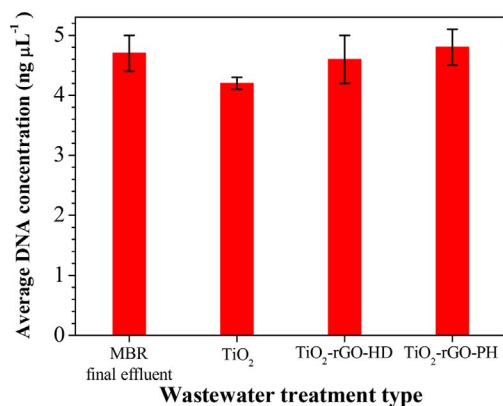


**Fig. 9.** The regrowth of total *E. coli* bacteria after incubation of treated samples at different experimental times at 37 °C for 24 h. The dotted line signifies the photocatalytic disinfection achieved reduction in *E. coli*. All bars above this limit indicate regrowth numbers (CFU mL<sup>-1</sup>) above the observed results during the photocatalytic experiments.  $C_r/C_t$  is the average CFU mL<sup>-1</sup> after 24 h of incubation (regrowth), divided by the average observed result during the photocatalytic experiments, from 3 replicate experiments ( $C_{cat} = 100 \text{ mg L}^{-1}$ ,  $V_{tot} = 300 \text{ mL}$ ).

On the other hand, as can be seen in Fig. 9, the photocatalytic treatment has achieved a reduced regrowth effect on *E. coli*. More specifically, the only  $C_r/C_t$  ratio that exceeded 1 was observed in the presence of TiO<sub>2</sub> at experimental times of 30 and 60 min ( $C_r/C_t$  ratio of 3 and 3.7 respectively). The other two photocatalytic materials achieved a reduction in bacterial counts, below a  $C_r/C_t$  ratio of 1. The most significant observation however, is that after 180 min of photocatalytic treatment, all three photocatalytic materials achieved a complete inactivation of *E. coli*, as no visible colonies were grown on the selective media. This result indicates the efficiency of photocatalytic oxidation in the permanent inactivation of *E. coli*. However, it was revealed that experimental treatment time also plays a significant role in the permanent inactivation of bacteria. The longer the treatment time, the more significant the inactivation effect exhibited on bacteria [83]. This indicates that, at the experimental conditions employed in the present work, 180 min is adequate treatment time to prevent regrowth of *E. coli*, even 24 h after treatment. However, it should be noted that in real-case wastewater treatment settings, the conclusions regarding the optimum time for complete absence of regrowth would vary, according to system volume, particle concentration and solar exposure time, amongst others.

### 3.7. Effect of photocatalysis on total DNA content

The total DNA content of samples was examined after photocatalysis in MBR effluent. The results are shown in Fig. 10. As can be seen, the



**Fig. 10.** The average DNA concentration (ng) in each µL of sample examined ( $C_{cat} = 100 \text{ mg L}^{-1}$ ,  $V_{tot} = 300 \text{ mL}$ ).

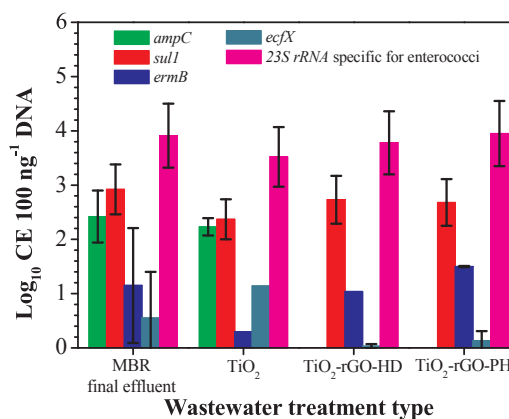
total genomic DNA concentration was not affected by the treatment, as the concentration remained steady with less than 10% change even after 60 min of photocatalysis. The genomic DNA concentration of the MBR effluent ( $4.7 \text{ ng } \mu\text{L}^{-1}$ ,  $\pm 6\%$ ) was not reduced by photocatalytic oxidation even though the total bacterial counts and the prevalence of ARB were influenced by the type of photocatalytic treatment (Sections 3.4 and 3.5). The total DNA content of the MBR effluent, treated with activated sludge, contains the genetic material contained intra- and extra-cellularly, and reflects the rich genetic diversity originating from the mixture of activated sludge acting on the micropollutants, with the incoming wastewater inside the reactor. The physically filtered MBR effluent contains a low concentration of total DNA, which may contain genetic material resistant to photocatalytic oxidation and to potential physical damage by catalyst particles. The potential genetic constituents of the genomic DNA may include genes conferring resistance to antibiotics and other antimicrobials, among others. As a result, to examine the molecular constitution of the remaining genomic DNA before and after photocatalytic treatment, the relative quantification of selected ARGs and species-specific genes took place next.

### 3.8. Development of species-specific genes and ARGs profiles

The prevalence of the selected ARGs (*ampC*, *sul1*, *ermB*) and species-specific sequences (*ecfX*, enterococcal 23S rRNA) was examined after photocatalytic treatment with the examined catalysts in the MBR effluent. The estimated log<sub>10</sub> cell equivalents (CE) 100 ng<sup>-1</sup> DNA in the examined samples during photocatalytic treatment for a total of 180 min are given in Fig. 11.

As can be seen, enterococci analysed with the 23S rRNA primer system was the most prevalent genetic sequence detected in all samples examined, ( $3.52\text{--}3.95 \text{ log}_{10} \text{ CE } 100 \text{ ng}^{-1} \text{ DNA}$ ), suggesting that their removal is more challenging compared to the removal of the other sequences examined. TiO<sub>2</sub> photocatalytic treatment displayed no reduction of *P. aeruginosa* (*ecfX*) ( $0.55\text{--}1.14 \text{ log}_{10} \text{ CE } 100 \text{ ng}^{-1} \text{ DNA}$ ), but the photocatalytic treatment with TiO<sub>2</sub>-rGO-PH and TiO<sub>2</sub>-rGO-HD successfully reduced *P. aeruginosa* in the respective wastewater effluent, thus indicating a sensitivity of the target organism to photocatalytic oxidation with the latter two synthesized photocatalysts, but not with pristine TiO<sub>2</sub>.

*Sul1* (sulfonamide resistance gene) is abundant after 60 min of treatment with TiO<sub>2</sub>, TiO<sub>2</sub>-rGO-PH and TiO<sub>2</sub>-rGO-HD photocatalysts ( $2.37\text{--}2.92 \text{ log}_{10} \text{ CE } 100 \text{ ng}^{-1} \text{ DNA}$ ), thus indicating that the bacterial carrier of the mentioned ARGs was relatively stable under photocatalytic treatment. *AmpC* (ampicillin resistance gene) abundance did not change after treatment with TiO<sub>2</sub>, but this specific gene encoding resistance to β-lactams was not detected in samples treated with TiO<sub>2</sub>-



**Fig. 11.** The log<sub>10</sub> abundance of the examined genetic parameters in the photocatalytically treated MBR effluent with the examined catalysts. Mean values are represented in log<sub>10</sub> cell equivalent (CE) values per 100 ng of DNA ( $C_{cat} = 100 \text{ mg L}^{-1}$ ,  $V_{tot} = 300 \text{ mL}$ ).

rGO-PH and TiO<sub>2</sub>-rGO-HD, indicating the superiority of the latter two photocatalysts in the removal of such resistant bacteria carrying the *ampC* gene. The erythromycin resistance gene *ermB* was also found in the MBR effluent (1.2. log<sub>10</sub> CE 100 ng<sup>-1</sup> DNA). The photocatalytic treatment achieved a 4-fold reduction in its prevalence after treatment (0.3 log<sub>10</sub> CE 100 ng<sup>-1</sup> DNA) with TiO<sub>2</sub> but it did not influence this target organism after treatment with TiO<sub>2</sub>-rGO-HD and TiO<sub>2</sub>-rGO-PH. The *mecA* gene was not detected in any of the samples examined.

The reduction in the abundance of *ampC* and *ecfX* can be attributed to intracellular DNA damages of bacteria produced by photo-irradiated TiO<sub>2</sub>-rGO and TiO<sub>2</sub> particles. According to Hirakawa et al. [81], photocatalytic treatment causes the breakdown of long DNA strands to shorter oligonucleotides, indicating the breakage of the deoxyribose phosphate backbone. Moreover, the authors suggest that DNA damage by photocatalytic treatment occurs at specific sites on the DNA double strand causing its photo-cleavage and causing base modifications, producing mutations in the offspring [82].

#### 4. Conclusions

The conclusions drawn from the present study can be summarized as follows:

- The synthesized photocatalysts were capable of removing the target antibiotics in real wastewater effluents under simulated solar radiation. TiO<sub>2</sub>-rGO-PH outperformed TiO<sub>2</sub> and TiO<sub>2</sub>-rGO-HD as to the removal of CLA and ERY, but TiO<sub>2</sub> was found to be the most efficient photocatalyst for the removal of SMX.
- Complete inactivation of *E. coli* after 180 min was exhibited with all examined photocatalysts, and antibiotic-resistant *E. coli* did not follow a particular inactivation trend, as each photocatalyst achieved a different inactivation pattern of *E. coli* resistant to each antibiotic.
- As treatment time increased, the cultivable *E. coli* counts decreased, and regrowth potential of the bacteria decreased, while it was shown that 180 min of photocatalytic treatment was adequate for the complete and non-reversible inactivation of *E. coli*, even after 24 h of post-treatment storage.
- The least amount of regrowth at all experimental times was observed in the presence of TiO<sub>2</sub>-rGO-HD, thus indicating its superiority to the other examined photocatalysts as regards permanent inactivation of bacteria leading to lack of repair/reactivation during storage of treated wastewater effluents.
- The examined genes, both species-specific and ARGs contained within these treated samples, did not all behave similarly during photocatalytic treatment, as specific genes were degraded, while others, such as *sul1*, *ermB* resistance genes, and enterococci detected via the 23S rRNA gene sequences, were persistent throughout the treatment.
- The existence of bacterial defense mechanisms against ROS-induced cellular damage may play an important part in the disinfection of both total and antibiotic-resistant *E. coli*, and this needs to be further investigated for the optimum disinfecting application of TiO<sub>2</sub>-rGO photocatalysis. Moreover, more studies are required in real wastewater effluents to determine the potential of graphene-based photocatalysts in the overall decontamination and disinfection of highly complex matrices, such as urban wastewaters.

Overall, it was shown through this study that the composition of the heterogeneous photocatalytic materials utilized has the potential to play an important role in the decontamination of wastewater from a mixture of antibiotic compounds, and disinfect such matrices from pathogenic bacteria and ARB. Of course, more studies are required in real wastewater to determine the potential of such materials in the overall decontamination and disinfection of highly complex matrices, such as urban wastewater. Finally, a complete and permanent

inactivation of ARB and the maximum removal of ARGs should be achieved to minimize the risk of antibiotic resistance spread into the receiving waters.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.apcatb.2017.11.020>.

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