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1 Combined photocatalytic degradation of pollutants and inactivation 2 of waterborne pathogens using Solar Light Active α/β -Bi₂O₃

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12 Abstract

13 A solar light active composite of α/β -Bi₂O₃ was synthesized using a chemical-free solid-state
14 reduction method. The obtained composite was characterized by X-ray diffraction, UV-Vis
15 spectroscopy, field emission scanning electron microscopy, and zeta potential. Initially, to validate
16 the photocatalytic effectiveness, the obtained α/β -Bi₂O₃ composite was used to degrade indigo
17 carmine dye. Then, the inactivation of *E. coli* and *S. aureus* waterborne pathogens was performed
18 on solid and in liquid media. On solid agar media, a significant inhibition zone was observed for
19 both bacterial strains. Similarly, in liquid culture, these strains *E. coli* and *S. aureus* were reduced
20 from 1×10⁶ CFU/mL to a few CFU/mL, after 240 min of photocatalytic exposure. Furthermore,
21 mixed wastewater of indigo carmine and *E. coli/S. aureus* were tested to study the combined
22 photocatalytic mechanism against the organic dye and microorganisms. Overall, the obtained
23 results suggested the efficacy of α/β -Bi₂O₃ towards visible light inactivation of bacteria even in
24 combination with other pollutants, highlighting the great potential of the advanced photocatalytic
25 process for combined treatment of organic pollutants and pathogens.

26 **Keywords:** Antibacterial, waterborne pathogens, α/β -Bi₂O₃, Photocatalysis, Wastewater.

1 **1. Introduction**

2 The anthropogenic and industrial activities produce in their wastewater (WW) high loads of
3 organic and inorganic compounds, such as dyes, resins, metals, nutrients and pathogenic bacteria,
4 as well as other by-products, classified as new emerging contaminants, like drugs and antibiotics
5 [1, 2]. The presence of these pollutants resulted in cross-contamination to surface water bodies
6 with organic and inorganic compounds alongside pathogens. For such a mixed stream, pathogens'
7 removal needs immediate attention due to the risk of waterborne diseases and the possibility of
8 microorganisms to develop antibiotic resistance [3, 4]. Many treatment systems and techniques are
9 available to remove pathogens from drinking water or WW, such as chlorination and ultraviolet
10 (UV) light [5, 6]. Chlorination is mostly applicable to all water systems, but it produces toxic by-
11 products, and some of them are carcinogens [7]. UV disinfection methods are widely used because
12 of their efficacy with a targeted attack on the DNA or RNA of the bacterial cells. However, the
13 UV treatment has some limitations, such as the high costs, the energy requirements, and the
14 reduced interactive exposure for bulk applications [8, 9].

15 Heterogeneous photocatalysis is an advanced oxidation process considered a green approach.
16 Indeed, it applies the irradiation and activation of photocatalytic materials via UV/solar light to
17 generate reactive oxygen species (ROS) for the degradation and treatment of contaminants, and
18 some studies reported as well a bacterial inactivation using photocatalytic metal oxide
19 nanoparticles (e.g. titanium, copper, and zinc-based) [10-12]. In principle, ROS attacks the cell
20 wall/membrane and results in bacterial cells' damage as revealed for instance by live/dead cell
21 viability using fluorescence microscopy [10, 11]. In a nutshell, all of these works highlight that
22 there is a constant need for efficient and stable photocatalytic active materials that could reduce
23 the water pollution problem and all of its consequences, including various diseases resulting from
24 pathogenic bacteria [11]. However, most of the mentioned studies focused on either antimicrobial
25 activity or the removal and degradation of organic/inorganic pollutants alone. Considering the
26 mixed nature of WW, rare work has been done on evaluating simultaneously the photocatalytic
27 antibacterial activity and organic pollutant degradation i.e. when the bacteria and organic
28 compounds are found together and interfering with each other.

29 Bismuth-based materials are widely studied for the photocatalytic degradation of organic
30 compounds [13-15]. Bismuth oxide (Bi_2O_3) has six polymorphic crystalline structures i.e. α , β , δ ,
31 γ , ϵ and ω : the α and β phases were mostly investigated for photocatalytic applications [15]. It has

1 been reported that Bi₂O₃ has better solar/visible light efficiency due to the wide absorption
2 spectrum in the visible region and optimum optical energy bandgap, if compared to other metal
3 oxides. For instance, compared to pristine TiO₂, the Bi₂O₃ photocatalytic activity is two times
4 higher under visible light [16].

5 In the present study, the use of solar light active α/β -Bi₂O₃ composite, earlier reported for
6 degradation of organic compounds [17], was investigated for bacterial inactivation alone or
7 combined with an organic dye, to simulate a real mixed WW. To the best of our knowledge this is
8 one of the first attempt for the combined degradation of organic pollutants and pathogens. The α/β -
9 Bi₂O₃ composite was synthesized by using a chemical-free solid-state thermal reduction method.
10 The synthesized bulk composite was characterized by X-ray diffraction (XRD) for the analysis of
11 crystal phase and composition, by UV-Vis spectroscopy for the optical properties, by scanning
12 electron microscopy to assess the morphological structure and zeta potential for the surface charge
13 properties. The obtained composite was initially tested for the photocatalytic degradation of indigo
14 carmine (IC) dye under visible light and afterward evaluated for the antibacterial potential against
15 two bacterial strains i.e. the Gram-positive *S. aureus* and the Gram-negative *E. coli*, by using the
16 plate count method and fluorescence microscopy.

17 **2. Material and methods**

18 **2.1 Materials**

19 Precursor salt of bismuth (Bi(NO₃)₃·5H₂O), triethylamine (TEA), p-benzoquinone (BQ),
20 isopropanol (IP) and Indigo Carmine (IC) were purchased from Sigma-Aldrich, Italy, and used as
21 received. Ethanol and sodium chloride (NaCl, 99.5%) were purchased from Daejung, China. For
22 the bacterial analysis, Luria-Bertani (LB) agar and broth and Tryptone Soya broth (TSB) were
23 purchased from Oxoid, England. The LIVE/DEAD® BacLight™ Bacterial Viability Kit was
24 purchased from Thermo Fisher Scientific, USA, for the live/dead cell staining.

25 **2.2 α/β -Bi₂O₃ synthesis**

26 The α/β -Bi₂O₃ composite was synthesized in bulk powder form, using a previously reported
27 method that employed thermal decomposition of Bi(NO₃)₃·5H₂O [17]. In brief, a measured
28 quantity of Bi(NO₃)₃·5H₂O salt was directly heated at 150 °C for 30 min in the muffle furnace i.e.
29 to evaporate the moisture content. Afterward, the temperature was increased to 250 °C and kept

1 for 2 hours. Finally, the resultant salt was calcined at 550 °C for 2 hours and allowed for ambient
2 cooling inside the furnace to obtain the thermally reduced and calcined bulk α/β -Bi₂O₃ composite.

3 **2.3 Characterization**

4 The as-calcined powder morphology was investigated through a MERLIN ZEISS field-emission
5 scanning electron microscopy (FESEM). The calcined powders were analyzed using X-ray
6 diffraction (XRD) to investigate both the crystalline structure and phase's composition. The UV-
7 Vis diffuse reflectance spectra were recorded by using a Varian Cary 5000 spectrophotometer
8 (Agilent Technologies). The Tauc plots of the Kubelka Munk function were used to calculate the
9 energy band gap (E_g) value. The Brunauer–Emmett–Teller (BET) specific surface area of the
10 obtained powder was measured through N₂ adsorption at 77 K on a Micromeritics Tristar-II
11 instrument. The zeta potential was measured through Malvern-Zetasizer, at neutral pH.

12 **2.4 Photocatalytic removal of indigo carmine**

13 To confirm the synthesized α/β -Bi₂O₃ photocatalytic activity, initially, the degradation of the IC
14 dye was evaluated under a white LED lamp (Phillips, emission spectrum ranging from 430 to 800
15 nm) with an irradiance of 100 W/m², placed 45 cm far from the dye solution. The IC dye
16 concentration was kept at 10 ppm, within a solution of 50 mL; the α/β -Bi₂O₃ loading in the dye
17 solution was 1 mg/mL (1:1). In the beginning, the suspension was stirred for 30 min in the dark
18 for adsorption-desorption equilibrium before exposure to irradiation. The dye solution's
19 absorbance spectra were recorded with different irradiation time intervals using a UV-Vis
20 spectrophotometer (Shimadzu 1800) by taking an aliquot of 3 mL centrifuged at 10,000 rpm for 3
21 min. The powder sample and aliquot were returned to the vial to preserve the same powder and
22 solution amount. The amount of IC dye removed during the treatment was estimated from the
23 decrease of the absorbance at 610 nm, corresponding to the principle peak in the spectra of the
24 dye. In order to confirm the dependence of ROS on the photocatalytic degradation of the dye, such
25 tests have been repeated in the presence of known ROS scavengers/quenchers (4% w/v) in the IC
26 dye solution: TEA for quenching h⁺, BQ for the reactive oxygen (O₂^{•-}) and IP for the OH[•] radicals.

27 **2.5 Preparation of Bacterial Culture**

28 Two bacterial strains were used to analyze the antibacterial activity by α/β -Bi₂O₃: *E. coli* (ATCC
29 8739), a Gram-negative considered an indicator of contaminations by bacteria in fresh/seawater

1 and *S. aureus* (ATCC 25923), a Gram-positive pathogenic bacterial strain generally found not only
2 in WW but even in hospital infections. *E. coli* was cultured in LB broth (Oxoid) [18], whereas *S.*
3 *aureus* was inoculated into TSB (Oxoid) [19, 20]. Afterward, both bacterial strains were grown
4 overnight in a shaker at 37 °C and 120 rpm. The day after, 50 mL of both strains' broth culture were
5 placed in the centrifuge tubes and centrifuged at 5000 rpm for 15 minutes. Bacterial biomass was
6 separated and washed several times with sterile 0.85% NaCl solution [21], then diluted in the same
7 saline solution to get a final bacterial suspension of 50 mL prepared at the concentration of 1×10^6
8 colony forming unit per mL (CFU/mL).

9 **2.6 Photocatalytic bacterial inactivation**

10 *2.6.1 Antibacterial tests on solid media*

11 The antibacterial tests of *E. coli* and *S. aureus* were conducted using a modified Kirby–Bauer test,
12 by placing the powders directly on agar without any filter disk in between. The bacterial inoculum
13 (100 μ L) at the concentration of 1×10^6 CFU/mL was spread on LB agar plates for both strains.
14 The test was then performed in the dark or under the same white LED lamp used for dye
15 degradation (see 2.4). In brief, 10 mg of α/β -Bi₂O₃ powder was placed in defined circular spots on
16 the bacteria inoculated petri dishes and then incubated overnight at 37 °C under dark conditions or
17 irradiated with the LED lamp. The zone of inhibition was calculated based on the method reported
18 in [22].

19 *2.6.2 Antibacterial tests in liquid media*

20 For photocatalytic evaluation of the inactivation of the same bacterial strains in liquid cultures, 50
21 mg of α/β -Bi₂O₃ powder was added to 50 mL of the above-mentioned bacterial suspension at a
22 concentration of about 1×10^6 CFU/mL. The resulted suspension (of α/β -Bi₂O₃ powder and
23 bacteria) was stirred in the dark for 30 minutes. Afterward, the photocatalytic response was
24 observed with and without the presence of α/β -Bi₂O₃ under the LED lamp at about 25 °C, following
25 the same conditions used for dye degradation (see 2.4). A “dark control” (the bacterial suspension
26 incubated with the nanomaterial in the dark), was also included. 100 μ L of microbial suspensions
27 were drawn from each tested condition after 30, 60, 120, 240 min, and were serially diluted.
28 Finally, 100 μ L of each serially diluted samples were spread on the respective agar plates and
29 incubated overnight at 37 °C for analyzing the reduction in bacterial growth respective to different

1 treatment time. Moreover, to analyze the stability and reuse potential of the recovered bismuth-
2 based nanomaterial, the tested α/β -Bi₂O₃ powder was recovered, washed, dried, and reused up to
3 3-cycles for antibacterial tests on solid media (as reported above).

4 2.6.3 Photocatalytic degradation of mixed pollutants and pathogens in an artificial WW

5 For the photocatalytic evaluation of an artificial WW i.e. containing IC dye and *E. coli* or *S. aureus*,
6 the stock solution was prepared by adding 5 ppm of IC in sterilized 0.85% NaCl. The overnight
7 grown culture of *E. coli* and *S. aureus* was centrifuged to separate the pellets from the broth. Then,
8 the separated pellets were washed with 0.85 % NaCl solution and resuspended in 50 mL of the
9 prepared stock solution of IC to make two mixed WW solutions i.e. one of IC and *E. coli*, and
10 another of IC and *S. aureus*. The initial concentration of bacteria in the mixed WW was maintained
11 at around 1×10^6 CFU/mL. For photocatalytic evaluation, the α/β -Bi₂O₃ powder was added in the
12 mixed WW, and the obtained slurry was initially stirred in the dark for 30 min. Afterward, the
13 irradiation was started, and the samples were collected after different exposure time, centrifuged,
14 and analyzed for removal of IC. The collected samples were serially diluted for bacterial reduction
15 analysis through the plate count method and live/dead cell staining using fluorescence microscopy
16 (see 2.7). All the experiments and analyses were performed at least twice for the reproducibility
17 of obtained results.

18 2.7 Fluorescence microscopy

19 During photocatalytic exposure, along with the bacterial density reduction analysis, the withdrawn
20 samples were analyzed for live/dead bacterial cell staining by using the LIVE/DEAD®
21 BacLight™ Bacterial Viability Kit and fluorescence microscopy. This kit includes the SYTO 9,
22 a dye that stains green on a fluorescence microscope in a live bacterial cell, and another dye,
23 propidium iodide (PI) stains red in case of a dead bacterial cell. The method for fluorescence
24 analyses was followed as per the guidelines provided by the viability kit supplier. In brief, 1 mL
25 of the treated suspension (i.e. a mixture of bacterias and α/β -Bi₂O₃) was drawn after 0, 30, 60, 120,
26 and 240 min, and centrifuged at $10,000 \times g$ for 10 minutes, then the supernatant was drained.
27 Further, the obtained bacterial biomass was washed with a sterile washing buffer solution,
28 centrifuged again, and resuspended and vortexed with 1 mL of 0.85% NaCl. Then, 3 μ L of the
29 mixed dye solution (PI: SYTO 9-1:1 (v:v)), was added to the resuspended solution, vortexed for
30 thorough mixing, and incubated for 15 minutes in dark conditions and at room temperature.

1 Afterward, 5 μL of the stained suspension was pipetted onto a sterilized glass slide and analyzed
2 on a Zeiss fluorescence microscope (Zeiss Axio Scope. A1 Carl Zeiss Germany). The FITC and
3 Texas Red filters were used for acquiring the stained live and dead cells images, respectively. The
4 acquired images of live and dead cells were analyzed using ImageJ 1.50d to assess the percentage
5 of live (green stained) and death (red stained) bacterial cells.

6 **3. Results and Discussion**

7 **3.1 Characterization of synthesized $\alpha/\beta\text{-Bi}_2\text{O}_3$**

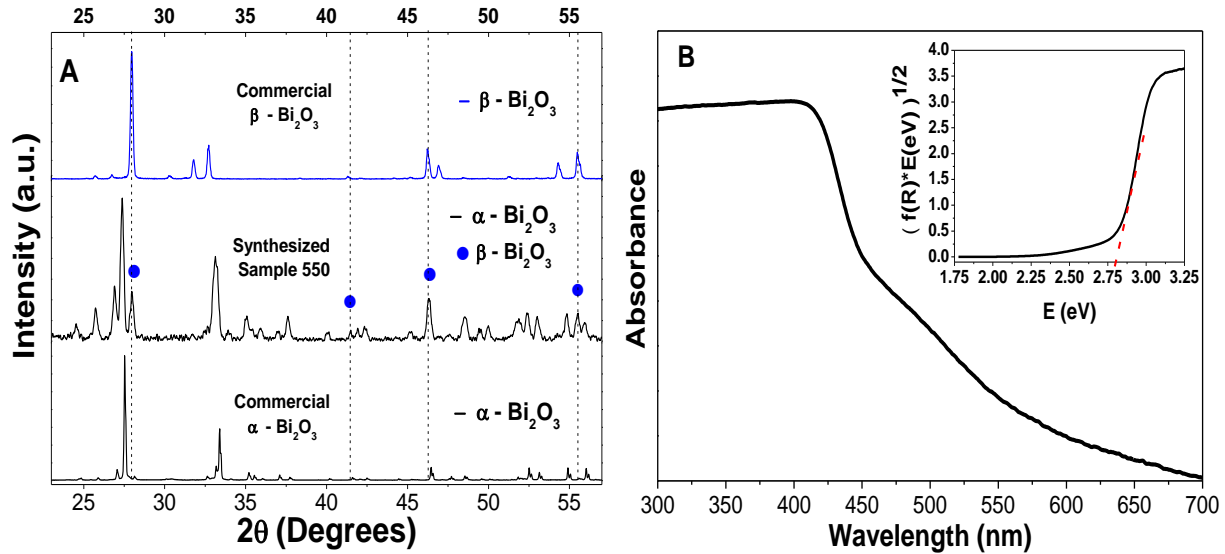
8 At first, the obtained powder was characterized using XRD, UV-Vis spectroscopy, FESEM, and
9 zeta potential to investigate the composition of the crystal phase and the optical, morphological,
10 and surface charge properties. Afterward, it was tested for photocatalytic removal of IC dye and
11 bacteria.

12 *3.1.1 XRD*

13 The XRD pattern of the as-synthesized powder sample at 550 $^\circ\text{C}$ and commercial $\alpha\text{-Bi}_2\text{O}_3$ and $\beta\text{-}$
14 Bi_2O_3 powders are shown in Fig. 1A. Most of the peaks correspond to monoclinic- $\alpha\text{-Bi}_2\text{O}_3$, with
15 principal peaks at 27.06, 27.52, 33.9 $^\circ$ (JCPDS card no. 01-071-0465). Additionally, some peaks
16 due to tetragonal $\beta\text{-Bi}_2\text{O}_3$ at 27.96, 41.36, 46.22 and 55.45 $^\circ$ (JCPDS card no. 01-078-1793) were
17 also found, showing the occurrence of a mixed composition of two different phases i.e. α and β
18 with the formation of an $\alpha/\beta\text{-Bi}_2\text{O}_3$ composite material of i.e. with around 20% proportion of $\beta\text{-}$
19 phase [17]. Most of the XRD peaks with minor intensity are ascribed to $\alpha\text{-Bi}_2\text{O}_3$ as revealed after
20 detailed analysis through the PDXL2 software and comparison to XRD patterns of the commercial
21 $\alpha\text{-Bi}_2\text{O}_3$ powders.

22 It is known that when the metastable $\beta\text{-Bi}_2\text{O}_3$ phase is cooled down from high temperatures to
23 ambient conditions, it is usually transformed into $\alpha\text{-Bi}_2\text{O}_3$ unless some dopants, such as Tantalum
24 or Niobium, are introduced to stabilize the metastable $\beta\text{-phase}$ at room temperature [23-25]. In one
25 of our studies [26], we established that the Nitrogen present in the precursor salt has a $\beta\text{-Bi}_2\text{O}_3$
26 stabilizing role. Indeed, the formation of $\alpha/\beta\text{-Bi}_2\text{O}_3$ can be associated with the decomposition of
27 the precursor salt ($\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$) at increased temperature, with a consequential loss of NO
28 and O_2 . With the complete decomposition of NO above 540 $^\circ\text{C}$ [26], some traces of N could remain
29 in the bulk synthesized Bi_2O_3 , stabilizing some of the $\beta\text{-Bi}_2\text{O}_3$ at room temperature (i.e. after most

1 of the transformation in α - Bi_2O_3) and resulting in the formation of a composite heterostructure of
2 α/β - Bi_2O_3 [17].



3
4 **Fig. 1 A) XRD pattern of synthesized bulk α/β - Bi_2O_3 , compared to commercial powders. B) UV-Vis**
5 **DRS analysis of the synthesized α/β - Bi_2O_3 , and the inset shows the Tauc plot obtained from Kubelka-**
6 **Munk method.**

7 3.1.2 Optical properties

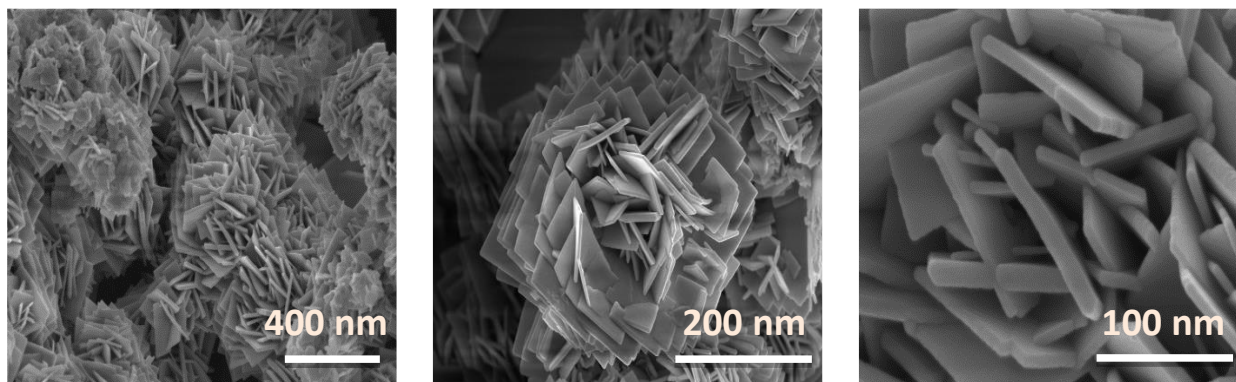
8 **Fig. 1B** shows the diffused reflectance UV-Vis spectroscopy (DRS) analysis of the synthesized
9 sample, and the inset shows the corresponding Tauc plot. The broad spectrum in the region from
10 400 to 450 nm is attributed to α - Bi_2O_3 , while the additional plateau i.e. from 470 to 550 nm
11 displayed the contribution of β - Bi_2O_3 [17]. In principle, these absorptions are associated with the
12 composite electronic transition from the valence band to the conduction band [17]. The estimated
13 bandgap energy of α/β - Bi_2O_3 was around 2.78 eV (shown in the inset **Fig. 1B**); the presence of the
14 heterojunction of β -phase influenced the slight reduction in the bandgap. Overall, considering the
15 band gap value and the corresponding optical transition in the visible region, these data confirm
16 that the nanomaterial can be fruitfully activated by visible light.

17 3.1.3 Morphology

18 **Fig. 2** shows the FESEM images of α/β - Bi_2O_3 at different magnification scales, presenting the
19 typical morphology of Bi_2O_3 materials with layered interconnected and flowery microstructure. A
20 similar morphology of bismuth oxides is also reported in various studies [27, 28]. The observed
21 thickness of the interconnected layers was around 30-45 nm. Interestingly, the tiny voids and

1 spaces between the interconnected layers revealed some macroporosity of the composite material,
2 facilitating interaction with contaminants for improved photocatalytic performance.

3



4

5 **Fig. 2 FESEM images of α/β -Bi₂O₃ at different magnification scales**

6 *3.1.4 Surface characteristics*

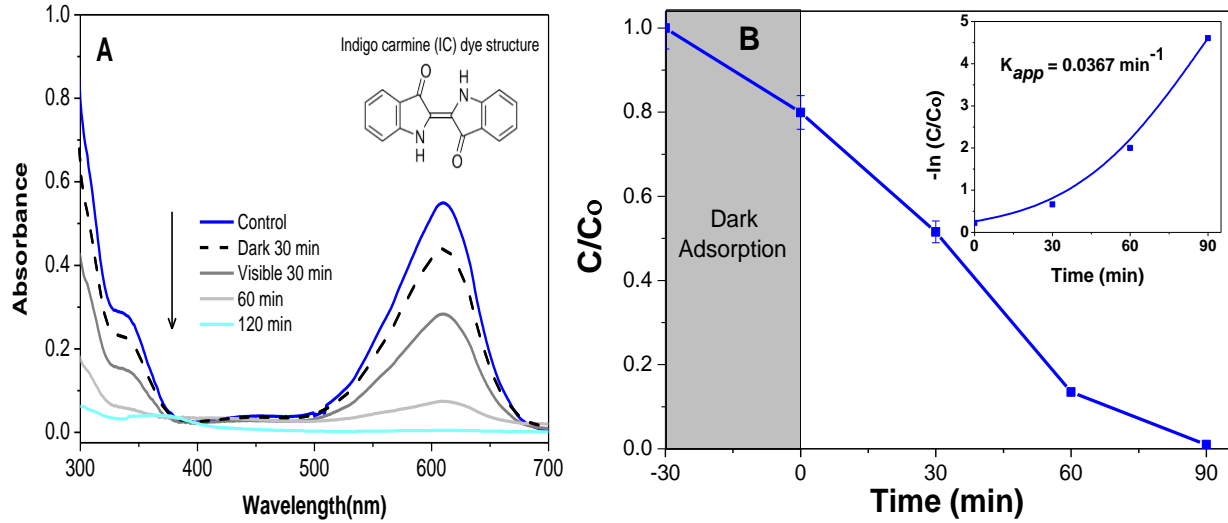
7 At neutral pH, the observed zeta potential of the α/β -Bi₂O₃ composite was around -35 mV,
8 suggesting that the negative net charge of the scattering sites around the composite surface could
9 facilitate the interaction with positively charged ions/molecules and, at the same time, could attain
10 better colloidal stability in a suspension [29]. In addition, the zeta potential was analyzed at
11 different pH; the obtained values were plotted and given in Fig. S1. In strong acidic conditions i.e.
12 at pH=2, the net negative charge of the α/β -Bi₂O₃ was stabilized to zero due to abundant
13 availability of H⁺ ions. On the contrary, at basic pH (=10), the negative ionic surface characteristic
14 was increased due to the abundance of OH⁻ ions on the α/β -Bi₂O₃ surface, reaching a value of
15 about -47 mV.

16 Moreover, the recorded BET specific surface area of the α/β -Bi₂O₃ composite was in the range of
17 7.6 m²/g, which suggests that the obtained composite is not porous and will allow minimum
18 adsorption on the surface or within the macropores (shown in FESEM images, Fig. 2).

19 **3.2. Photocatalytic removal of Indigo Carmine (IC)**

20 Fig. 3A shows the absorbance spectrum of the IC dye at different irradiation times in the presence
21 of the α/β -Bi₂O₃ composite under visible light, while Fig. 3B shows the relative concentration and
22 kinetic profile of IC dye. The obtained kinetics revealed that IC's removal follows a 1st order linear
23 degradation kinetics and a calculated kinetic apparent rate (K_{app}) of $3.67 \times 10^{-2} \text{ min}^{-1}$. In our earlier

1 work [17, 30], the IC dye's degradation mechanism was explained in detail; in brief, the oxidation
2 species attacked the IC dye and dissociated into amine-sulfo-benzoic acid and isatin sulfonic acid
3 and partially mineralized to carboxylic groups and phenol derivatives [30-32].



4

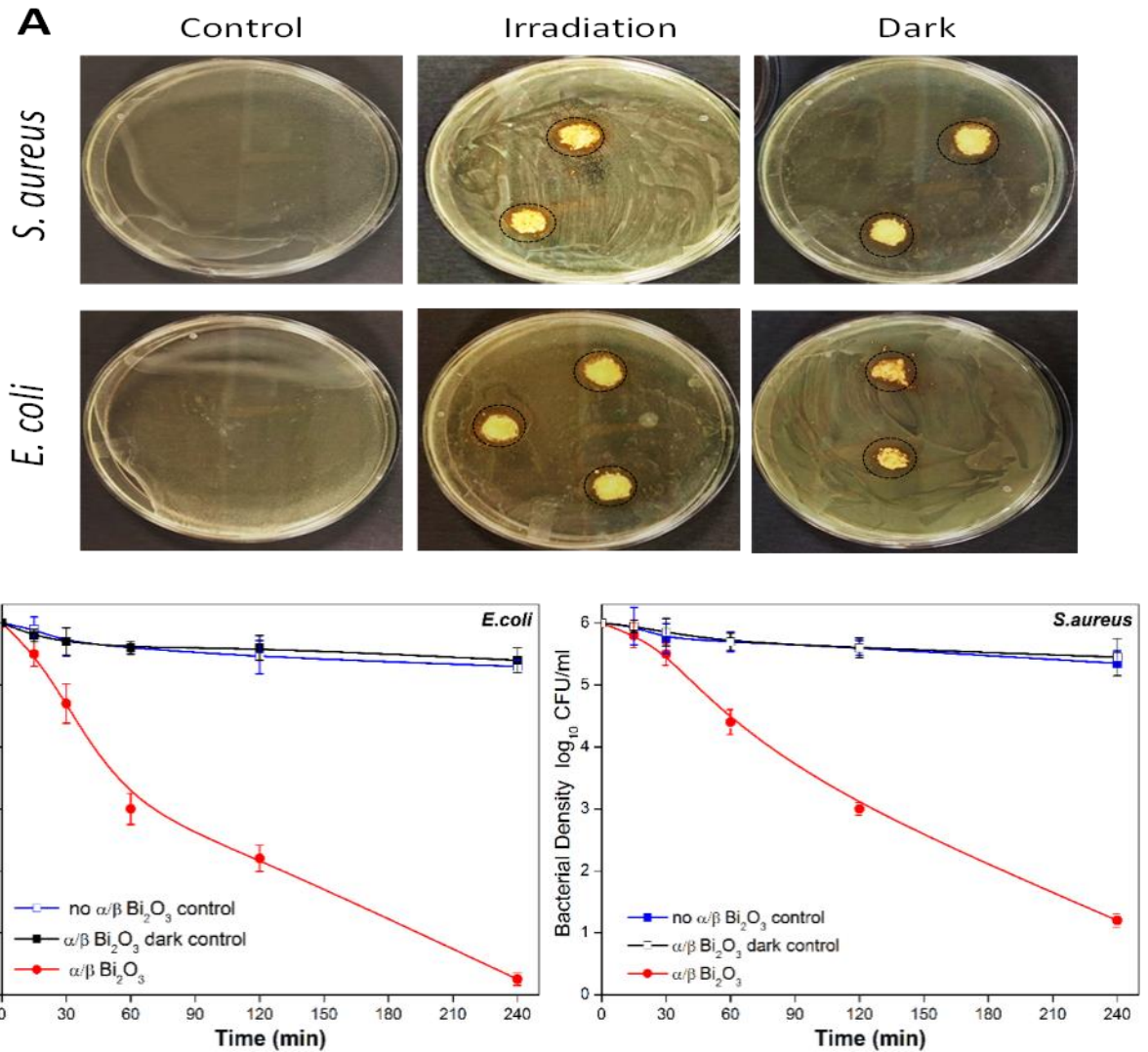
5 **Fig. 3 A) Absorbance spectra of the IC dye removal using α/β -Bi₂O₃ under LED lamp; B) relative**
6 **concentration decrease at different treatment time (considering the optical density at 610 nm; each**
7 **point is the average of two replicates); inset: kinetic curve plot and calculated kinetic apparent**
8 **constant (K_{app}).**

9 3.3 Photocatalytic bacterial inactivation on solid and liquid media

10 Once the composite was proved to be effective towards an organic dye, the main focus was to
11 evaluate the photocatalytic inactivation of bacteria using the α/β -Bi₂O₃ composite. Therefore, two
12 pathogen indicators (commonly found in drinking water) i.e. *E. coli* and *S. aureus* were selected.
13 The observed inhibition halo for both bacterial strains is shown in Fig. 4A. Compared to the biotic
14 control (without the presence of α/β -Bi₂O₃), the petri dishes with the presence α/β -Bi₂O₃ showed
15 growth inhibition of both *E. coli* and *S. aureus* under the LED light irradiation, showing an
16 inhibition halo for *E. coli* and *S. aureus* of around 11 mm and 10 mm, respectively. These results
17 revealed the potential of α/β -Bi₂O₃ towards inhibition of both bacterial strains.

18 Due to the well-known limitations (e.g. mass transfer limitations, absorption, and complexation to
19 organic compounds) that affect metal bioavailability during tests on solid media [33], the
20 effectiveness of the α/β -Bi₂O₃ composite was evaluated in liquid cultures, too. The obtained
21 results, obtained by assessing the number of CFU/mL (shown in Fig. 4B), confirmed the bacterial
22 cell density reduction for both strains after 240 min of photocatalytic exposure. Indeed, the initial

1 bacterial density (1×10^6 CFU/mL for both strains) was significantly reduced (about 99.99%
 2 reduction) for both *E. coli* and *S. aureus*, respectively. However, the reduction was found higher
 3 in the case of *E. coli*. A “dark control” in which the bacterial suspension was mixed with the
 4 nanomaterial but incubated in the dark, was also included for each strain. The cell densities
 5 recorded during time for these dark controls were comparable to those obtained by the controls
 6 cultured under the visible light, but in the absence of the α/β - Bi_2O_3 composite.



7

8 **Fig. 4 A)** Photocatalytic inhibition of *E. coli* and *S. aureus* on solid media in the presence of α/β - Bi_2O_3
 9 under visible light irradiation; **B)** Bacterial density (CFU/mL) reduction of *E. coli* (EC, left) and *S.*
 10 *aureus* (SA, right) liquid suspensions incubated under visible light with and without (control) the
 11 presence of α/β - Bi_2O_3 . A “dark control” (bacterial suspension incubated with the nanomaterial in the
 12 dark), was also included.

3.4 Fluorescence microscopy of live/dead bacterial cells

To confirm if the α/β - Bi_2O_3 has a biocidal effect on the bacterial strains, live/dead staining was adopted in combination with fluorescence microscopy. The fluorescent green-stained cells represent live cells, whereas the red-stained represent dead ones. The acquired merged live/dead fluorescence microscopy images of *E. coli* (top), and *S. aureus* (bottom) are shown in Fig. 5, while the separate live and dead stained cells are shown in supplementary data Fig. S2. For both strains, in the beginning, plenty of live green-stained cells could be observed, even if the number of dead cells (red-stained) start to increase already after 15 minutes for both strains. In contrast, at increasing photocatalytic exposure, the bacterial cells in the presence of α/β - Bi_2O_3 resulted in the cell membrane's rupture, as indicated by the increasing number of red stained cells during the treatment. The increased appearance of red-stained cells at longer exposures revealed that α/β - Bi_2O_3 , along with growth inhibition, could kill both bacterial strains. Potentially, during the photocatalytic reaction, the nanomaterial produces ROS that could attack the bacterial cell and leads to bacterial cell damage that increased the permeability of the membrane to PI staining [11].

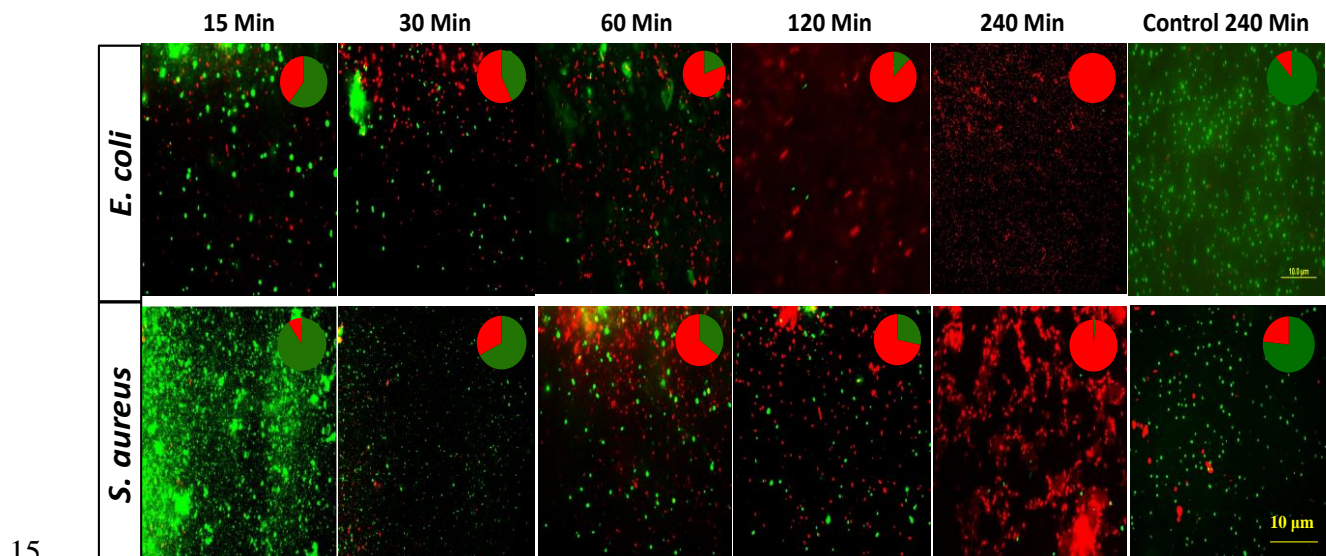


Fig. 5 Merged live/dead fluorescence microscopy images of *E. coli* and *S. aureus* at different treatment times: 15 min, 30 min, 60 min, 120 min, 240 min, and images of controls (without α/β - Bi_2O_3) after 240 min.

Compared to the obtained results in Fig. 4B, the fluorescence images in Fig. 5 confirmed a similar trend of higher inactivation of *E. coli* cells than the *S. aureus*. For instance, by comparing the values obtained after 60 minutes of treatments, the higher inactivation of *E. coli* result significant

1 if compared to that of *S. aureus* (p value= 0.0467). The estimated proportions of live and dead
 2 cells of both strains i.e. after prolonged photocatalytic exposure of 240 min, are given in Table 1,
 3 which again revealed an increased proportion of dead cells over live in the case of *E. coli*. Anyway,
 4 for both strains, the time required for the complete sanitation of water can be compatible with
 5 standard WW treatments.

6 **Table 1. Proportions of live and dead bacterial cells at different treatment times.**

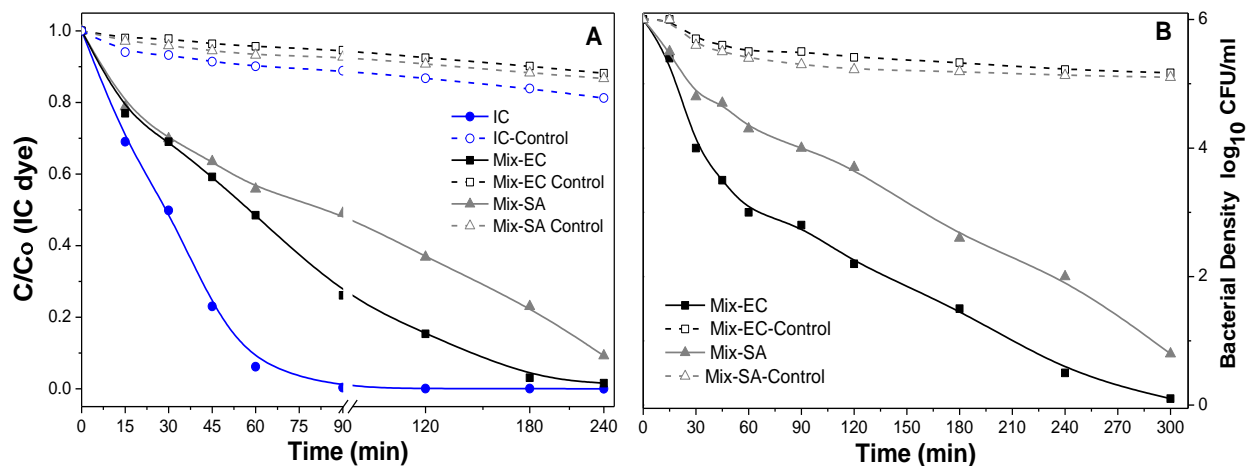
Time (min)	<i>E. coli</i>		<i>S. aureus</i>	
	Live %	Dead %	Live %	Dead %
15	60.24 ± 4.07	39.76 ± 1.23	90.56 ± 12.89	9.44 ± 1.74
30	43.04 ± 3.32	56.96 ± 1.40	67.19 ± 9.21	32.81 ± 5.27
60	19.12 ± 1.94	80.88 ± 1.69	35.47 ± 1.28	64.53 ± 4.19
120	12.33 ± 0.18	87.67 ± 5.19	28.31 ± 1.05	71.69 ± 3.19
240	0.33 ± 0.06	99.67 ± 14.47	1.90 ± 0.02	98.10 ± 2.42
Control*	88.96 ± 0.76	11.04 ± 0.25	77.12 ± 2.35	22.88 ± 0.63

7 * The control corresponds to a bacterial suspension irradiated with visible light for 240 minutes,
 8 without the photocatalyst's presence.

9 **3.5 Photocatalytic degradation of mixed pollutants and pathogens in an artificial WW**

10 To investigate the photocatalytic response towards IC and bacteria's combined presence, mixed
 11 WW of IC and *E. coli/S. aureus* were tested with and without the presence of α/β -Bi₂O₃. The
 12 obtained results of this artificial WW for both bacterial strains are shown in Fig. 6. As compared
 13 to the previous results of IC removal alone, the removal kinetics of IC was reduced in the mixed
 14 WW i.e. from $3.67 \times 10^{-2} \text{ min}^{-1}$ to $0.8 \times 10^{-2} \text{ min}^{-1}$, as estimated from the relative concentration
 15 decrease in Fig. 6A. The decreased kinetics was probably due to the relative optical density of
 16 bacterial suspension that hindered the irradiation passage and α/β -Bi₂O₃ activation, and besides,
 17 limited reactive species that could have simultaneously targeted both the bacteria and IC dye
 18 molecule and reduced the overall kinetics. Further, Fig. 6B shows the control of both mixed WW
 19 solutions i.e. with and without the presence of α/β -Bi₂O₃, the bacterial growth of both bacteria
 20 remained unaffected due to the presence of IC. Compared to the previous results (Fig. 4B), almost
 21 a similar bacterial density reduction trend was observed for both bacterial strains. The inactivation

1 was more effective towards *E. coli* than *S. aureus*. The control of all the mixed WW showed little
 2 change in either removing IC (alone and in the mixed WW) or in the bacterial density after
 3 prolonged exposure of irradiation under LED lights if the composite was not added. The overall
 4 results revealed that the presence of bacteria interfered with the photocatalytic degradation and
 5 removal of the organic compound, as observed by reduced IC dye kinetics in the mixed WWS. On
 6 the contrary, the inactivation of both bacterial strains was unaffected in the presence of IC, as the
 7 observed density reduction trend was similar compared to the bacterial suspension without the
 8 presence of IC dye.



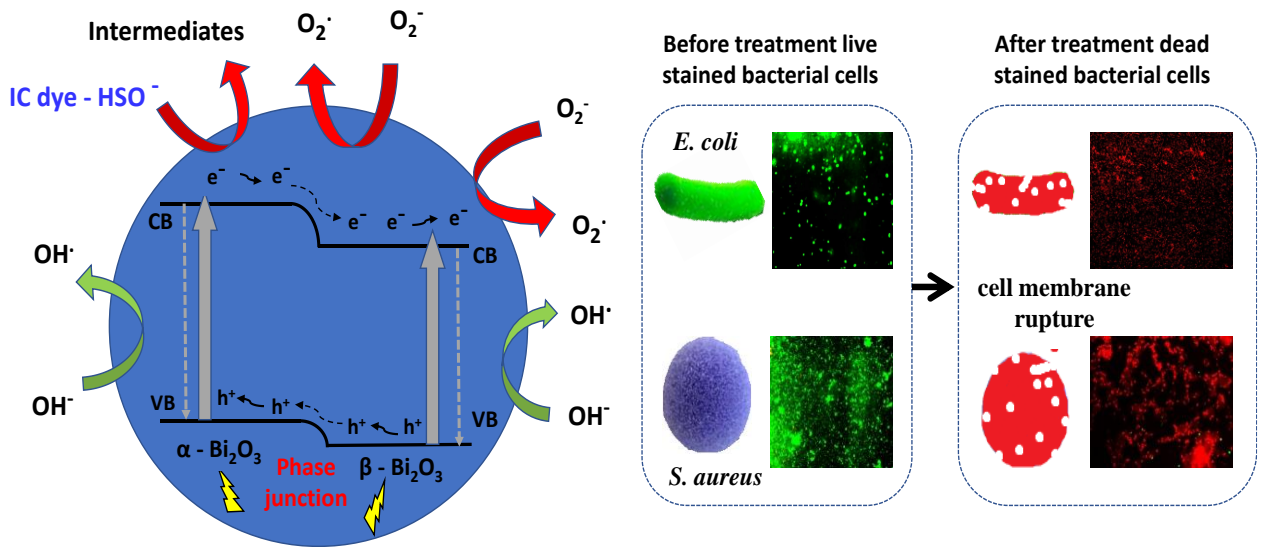
10 **Fig. 6. A) Relative concentration decrease of IC alone and in mixed wastewater (WW) with *E. coli*/*S.***
 11 ***aureus* with and without the presence of α/β - Bi_2O_3 ; B) Bacterial density reduction of both *E. coli* and**
 12 ***S. aureus* in mixed WW with IC with and without the presence of α/β - Bi_2O_3 (Mix-EC referred to a**
 13 **mixed WW of IC and *E. coli*, Mix-SA is referred to a mixed WW of IC and *S. aureus*.**

14 3.6 Stability and reuse of α/β - Bi_2O_3

15 To evaluate the α/β - Bi_2O_3 powder's reuse and stability potential, it was recovered, washed, dried,
 16 and reused for up to 3-cycles, for antibacterial tests on solid media and to observe any change in
 17 photocatalytic response. The observed inhibition zone on inoculated petri dishes in the presence
 18 of the recovered α/β - Bi_2O_3 powder, shown in supplementary data Fig. S3, revealed the stability
 19 and reusability of α/β - Bi_2O_3 for subsequent cycles of photocatalytic inactivation. This result
 20 highlight that this nanomaterial can be used more than one time, which is an advantage in the case
 21 of WW plants.

3.7 Photocatalytic mechanism

From the overall results, the encountered photocatalytic mechanism of action of the α/β - Bi_2O_3 powder can be explained. Previously, we reported that ROS were generated on the α/β - Bi_2O_3 surface due to resultant reactions of the photogenerated electrons (e^-) and holes (h^+) with the dissolved O_2 and OH^- radicals [17]. The cascade of photogenerated e^- and holes h^+ shown in Fig. 7 i.e., photocatalytic induced reactions, was facilitated due to heterojunction between α and β phases [17].



8

9 **Fig. 7. Mechanism of origination of reactive species at α/β - Bi_2O_3 surface, degradation of IC dye, and**
10 **inactivation of *E. coli* and *S. aureus* bacterial cells.**

11 To further explore the photocatalytic activity and proof the oxidative/reductive paths, we did some
12 photocatalytic tests with and without the addition of some reagents for the scavenging/quenching
13 of holes and ROS. Indeed, some researchers have used TEA for quenching h^+ , BQ for the reactive
14 oxygen ($\text{O}_2^{\cdot -}$) and IP for the OH^{\cdot} radicals [27, 34, 35], to observe any significant change in the
15 degradation kinetics due to their presence in the dye solution. The addition of quenchers could
16 influence the photocatalysis process i.e. by reducing the dye degradation kinetic rate because of
17 the quenching of the generated reactive species or holes around the photocatalyst surface.
18 Therefore, the degradation of the IC dye solution (10 ppm) was investigated under visible light,
19 using α/β - Bi_2O_3 , with and without the presence of these quenchers i.e. TEA, BQ, and IP.

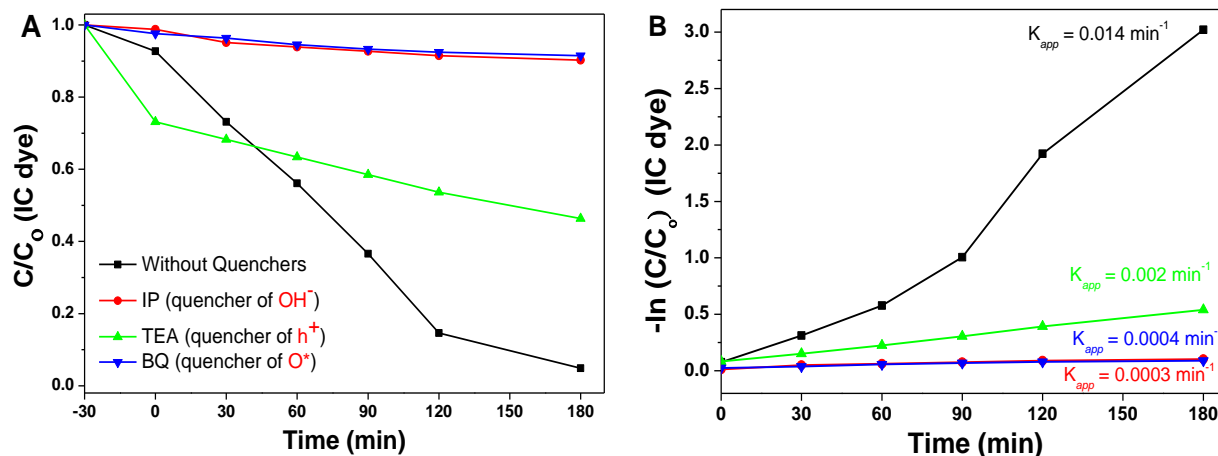


Fig. 8 A) Relative concentration of IC with and without quenchers; B) kinetic curves and K_{app} .

Fig. 8 show the C/C_0 plots, and the kinetics curves along with the determined K_{app} of IC dye degradation with and without the quenchers in solution. It was observed that the degradation rate was drastically reduced in the presence of each quencher compared to the results without their addition, which revealed that both the $O_2^{\bullet-}$ and OH^{\bullet} radicals were originated from the α/β - Bi_2O_3 surface and were responsible for the removal of the IC dye. Moreover, this also supports that the electrons are well separated from the h^+ in generating the reactive species quenched with quencher addition and decreasing degradation kinetics. The ROS originated on the α/β - Bi_2O_3 surface, probably could have degraded the organic dye, as well as the bacterial membrane/cell-wall [17, 36]. In the case of organic dyes, these reactions are responsible for partial or complete degradation of the parent molecule i.e. with the formation of intermediates and mineralization into CO_2 , H_2O , NH_3 etc. [30, 36]. Instead, in the case of bacteria, the ROS, and in particular the hydroxyl radicals ($^{\bullet}OH$), the superoxide ($O_2^{\bullet-}$) and H_2O_2 originated by the photocatalytic reactions probably attacked the cell-wall/membrane and their constituents, resulting in different kinds of damage, as shown in the scheme given in Fig. 7. Indeed, it is widely known that these molecules can cause the peroxidation of lipids and phospholipids, as well as the oxidation of proteins present on the membranes [37]. These reactions can disrupt the membrane itself or simply its lipid bilayer organization, eventually leading to the efflux of cytosolic contents or cell lysis. Indeed, ROS have potent antimicrobial activity against bacteria, fungi and viruses, due to their quick action against various Gram-positive and Gram-negative bacteria, including multidrug-resistant strains [38]. This mechanism of action has been suggested for well-known metal such as silver [39], or other metal

1 oxides applied as antimicrobial [19]. Moreover, it is consistent with the earlier reported results of
2 the efficacy of β - Bi_2O_3 on *E. coli* and *S. aureus* [21]. These strains belong to different microbial
3 groups, namely Gram-negative and Gram-positive, and have different characteristics of cell
4 wall/membrane thickness, morphology, and chemical groups [40]. Therefore, both bacteria's
5 photocatalytic rupture could have occurred differently, as reported for TiO_2 [41]. The Gram-
6 negative bacteria like *E. coli* has an outer membrane rich of lipopolysaccharides and
7 phospholipids, a thin layer of peptidoglycan, and an inner phospholipidic membrane. Whereas,
8 Gram-positive bacteria, like *S. aureus*, have a thick cell wall of peptidoglycans that covers the
9 cytoplasmic membrane. Furthermore, it is well-known that bacteria belonging to *Staphylococci*
10 groups can actively produce catalase, a heme protein enzyme that decomposes hydrogen peroxide
11 to water and oxygen, protecting the cell from hydrogen peroxide-mediated leukocyte bactericidal
12 mechanisms [42]. Moreover, *Staphylococci* can usually produce exopolysaccharides that could
13 increase the resistance against the antibiotics and antimicrobial agents [43]. All of these
14 considerations match with the obtained results. The high proportion of dead (red-stained) cells for
15 both the treated strains suggested that the originated ROS could attack and inactivate both
16 microorganisms through the cell wall/membrane's rupture. At the same time, *E. coli* was
17 inactivated more rapidly than *S. aureus*, which appeared to be more resistant to the ROS originated
18 by the α/β - Bi_2O_3 composite's photocatalytic action.

19 **Conclusion**

20 The α/β - Bi_2O_3 composite was obtained through facile solid-state thermal reduction of bismuth
21 nitrate salt at 550 °C. The obtained composite with a balanced proportion of α -phase and the β -
22 Bi_2O_3 phase contributed to the effective removal of IC dye and inactivation of Gram-negative *E.*
23 *coli* Gram-positive *S. aureus*. In the presence of α/β - Bi_2O_3 , the significant inhibition zone on the
24 solid bacterial culture was observed, and bacterial cell density reduction in liquid suspension was
25 achieved. The live/dead fluorescence microscopy analyses of the treated bacterial suspension
26 revealed an almost complete biocidal effect after the prolonged photocatalytic exposure up to 240
27 min. Further, the photocatalytic evaluation of the mixed WW of IC and *E. coli/S. aureus* revealed
28 that the removal kinetics of IC was affected and reduced due to interference of both bacteria, while
29 the photocatalytic inactivation of both strains remained unaffected even with the presence of IC.
30 Finally, even if a detailed photocatalytic investigation must be considered, the presented data

1 revealed that α/β -Bi₂O₃ was able to attack *E. coli* and *S. aureus* and showed the potential for the
2 combined treatment of organic pollutants and microbial pathogens.

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