



Basic physiology of *Pseudomonas aeruginosa* contacted with carbon nanocomposites

Adrian Augustyniak^{1,2,3} · Kamila Dubrowska² · Joanna Jabłońska² · Krzysztof Cendrowski⁴ · Rafał J. Wróbel² · Mateusz Piz² · Elżbieta Filipek² · Rafał Rakoczy²

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Abstract

Experiments describing properties of nanomaterials on bacteria are frequently limited to the disk diffusion method or other end-point methods indicating viability or survival rate in plate count assay. Such experimental design does not show the dynamic changes in bacterial physiology, mainly when performed on reference microorganisms (*Escherichia coli* and *Staphylococcus aureus*). Testing other microorganisms, such as *Pseudomonas aeruginosa*, could provide novel insights into the microbial response to nanomaterials. Therefore, we aimed to test selected carbon nanomaterials and their components in a series of experiments describing the basic physiology of *P. aeruginosa*. Concentrations ranging from 15.625 to 1000 µg/mL were tested. The optical density of cultures, pigment production, respiration, growth curve analysis, and biofilming were tested. The results confirmed variability in the response of *P. aeruginosa* to tested nanostructures, depending on their concentration. The co-incubation with the nanostructures (in concentration 125 µg/mL) could inhibit the population growth (in most cases) or promote it in the case of graphene oxide. Furthermore, a specific concentration of a given nanomaterial could cause contradictory effects leading to stimulation or inhibition of pigmentation, an optical density of the cultures, or biofilm formation. We have found that particularly nanomaterials containing TiO₂ could induce pigmentation in *P. aeruginosa*, which indicates the possibility of increased virulence. On the other hand, nanocomposites containing cobalt nanoparticles had the highest anti-bacterial potential when cobalt was displayed on the surface. Our approach revealed changes in respiration and growth dynamics that can be used to search for nanomaterials' application in biotechnology.

Keywords Graphene oxide · Hybrid nanostructures · Bacterial physiology · Stimulation · Toxicity · Microorganisms

Introduction

Usually, nanomaterials are studied on microbiological models to prove their toxicity (Maurer-Jones et al. 2013b; Käkinen et al. 2016). Undeniably, nanostructures may express anti-bacterial potential (Sirelkhatim et al. 2015; Mosselhy et al. 2017). However, the effects are in most cases described only with viability measures, often restricted to plate count methods or optical density measurements (Sikora et al. 2016, 2017). This approach may suggest a decrease in viability, although it does not describe the changes in bacterial physiology (Augustyniak et al. 2019). Therefore, the literature also argued that it is necessary to propose a comprehensive approach for studying nanomaterials on microorganisms which will broaden the current perspective, frequently restricted to solely studying the toxicity (Nawrotek and Augustyniak 2015). Additionally, the antimicrobial activity of nanomaterials is typically studied in end-point assays that

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✉ Adrian Augustyniak
adrian.augustyniak@zut.edu.pl

- 1 Chair of Building Materials and Construction Chemistry, Technische Universität Berlin, Berlin, Germany
- 2 Faculty of Chemical Technology and Engineering, West Pomeranian University of Technology, Al. Piastow 42, 71-065 Szczecin, Poland
- 3 Faculty of Biotechnology and Animal Husbandry, West Pomeranian University of Technology, Szczecin, Poland
- 4 Faculty of Civil and Environmental Engineering, West Pomeranian University of Technology, Szczecin, Poland

show the final result of culture (Gopal et al. 2013). Even if the cultures are inhibited during the initial stage, the surviving population may regrow and reach a level similar to the control (Sikora et al. 2018).

Another limiting factor in the current state of nanomaterial–bacteria interactions is the selection of bacteria for studies (Sikora et al. 2018). Usually, model bacteria such as *Escherichia coli* or *Staphylococcus aureus* are used for this purpose (Ortiz and Torres 2014). While these microorganisms are good models for comparative studies regarding the antimicrobial activity of a compound, their value for biotechnological studies can be surpassed by other microorganisms. For that reason, the inclusion of *Pseudomonas aeruginosa* and other pseudomonads was also proposed to serve as additional models that have high medical and biotechnological significance (Augustyniak et al. 2020a; Roszak et al. 2021). This non-fermenting rod-shaped bacterium expresses high adaptability to the surrounding environment and is ubiquitous in soil, water, and hospital wards (Palleroni 2015). Infections caused by *P. aeruginosa* can be challenging to treat because of its quickly arising antibiotic resistance (Pang et al. 2019). From the biotechnological standpoint, this microorganism is a potent producer of demanded metabolites such as phenazines or rhamnolipids (Pierson and Pierson 2010; Lotfabad et al. 2013). It has been shown that pseudomonads may react differently to nanomaterials than *E. coli* and *S. aureus*, potentially modulating their virulence, e.g., by increasing biofilm production or secretion of pigments (Wang et al. 2016). To that extent, testing nanomaterial on the *P. aeruginosa* model can reveal the possible response of this microorganism to therapy or biotechnological processes.

As shown above, the use of pseudomonads to study the activity of nanomaterials is justified and encouraged. Therefore, the study aimed to record several physiological characteristics of *P. aeruginosa* in response to selected nanomaterials. An additional goal was to assess the planned

experimental approach in the scope of basic physiological features of the selected microorganism.

Materials and methods

Materials

Pseudomonas aeruginosa ATCC®27853™ from the Department of Chemical and Process Engineering collection at the West Pomeranian University of Technology in Szczecin was used as biological material for the experiments.

The research material consisted of eight nanomaterials indicated in Table 1. The central nanomaterial for the experiments was CNT/CoOF, a hybrid nanocomposite containing carbon flakes obtained with cobalt MOF and multi-walled carbon nanotubes on the surface. The characterization of the nanomaterials was described in previous publications. A detailed description of the synthesis and physicochemical characterization of these nanomaterials is provided in the Supplementary material.

Methods

Preparation of biological material

Bacteria were kept on beads (Microbank® system, Biomaxima, Lublin, Poland) at – 21 °C. Before use, cells were revived on trypticasein soy agar (TSA, Biomaxima, Lublin, Poland) and incubated at 37 °C for 14–16 h. All overnight cultures in a liquid medium were prepared by transferring a single colony to 30 mL of trypticasein soy broth (TSB, Biomaxima, Lublin, Poland) medium and subsequent incubation at 37 °C for 14–16 h. All inocula for the experiments were prepared by diluting the overnight culture in ratio 1:500 with fresh TSB and gentle shaking for an even dispersion of cells in the medium. In the experiments requiring twofold

Table 1 Nanomaterials used in this study

No.	Full name	Short name	Reference
1	Graphene oxide	GO	Asadi et al. (2015)
2	Titanium dioxide particles	TiO ₂	Rejek et al. (2021)
3	Carbon nanotubes	CNT	Cendrowski et al. (2014)
4	Carbon nanotubes functionalized with titanium dioxide particles	CNT/TiO ₂	Cendrowski et al. (2014)
5	Carbonized metal–organic framework based on cobalt, after hydrochloric acid purification	CoOF	Cendrowski et al. (2017)
6	Carbonized cobalt–organic framework with cobalt particles located on their surface	CoOF/Co	Cendrowski et al. (2017)
7	Carbonized cobalt–organic framework functionalized with titanium dioxide particles	CoOF/TiO ₂	Unpublished material (more detail in the Supplementary information)
8	The hybrid structure of carbonized cobalt organic framework and carbon nanotubes	CNT/CoOF	Cendrowski et al. (2021)

dilutions with nanomaterial suspension, inocula were prepared in twice concentrated TSB.

Preparation of nanomaterials for biological studies

Stock solutions of nanomaterials were prepared by dispersing 2 mg of nanomaterial in 1 mL of deionized water in 1.5 mL Eppendorf-type tubes. Subsequently, solutions were heated at 100 °C for 10 min to sterilize them and sonicated (35 kHz) in an ultrasonic bath sonicator for 45 min. Before use in every experiment, the suspensions were additionally sonicated for 15 min.

Optical density, fluorescence, and respiration after 24 h

The cultures were prepared in 96-well transparent (F-type) polystyrene plates. In the first step, nanomaterials were prepared. The stock suspensions were diluted in a series of twofold dilutions. In total, seven concentrations of nanomaterials (15.625–1000 µg/mL) and a control sample containing deionized water were tested. Afterward, the inoculum prepared in 2 × TSB was added. The final volume in each well was 100 µL. The plates were incubated at 37 °C with agitation (140 rpm). Each case was studied in eight repetitions. After 24 h incubation, the optical density ($\lambda = 600$ nm) was measured on spectrophotometer BioTek Synergy H1 (BioTek, Winooski, VT, USA).

The same instrument also served in the fluorescence and respiration measurements. The fluorescence of the cultures was measured with $\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{em}} = 520$ nm.

The respiration scan was performed with a resazurin assay in the following manner. Each well was loaded with 10 µL of resazurin (1 mg/mL in PBS), and the fluorescence ($\lambda_{\text{ex}} = 520$ nm and $\lambda_{\text{em}} = 590$ nm) was scanned for 4 h with reads in 15 min intervals. The resulting curves were fitted to a logistic function in Origin 2021 software (OriginLab, Northampton, MT, USA).

Growth curves

Cultures were set up in 24-well flat-bottomed polystyrene plates. The total volume in each well was 1 mL composed of 900 µL of overnight culture diluted in TSB medium in ratio 1:500 and 100 µL of the tested nanomaterial suspension. The optical density ($\lambda = 600$ nm) was monitored every 30 min for 16 h at 37 °C. Positive (bacteria without nanomaterial) and negative controls containing TSB or ultrapure water were included in all experiments. Each case was prepared in four repetitions to minimize the influence of nanomaterial agglomerates on the reads. Obtained curves were used as an input to fitting in Origin 2021 software. Afterward, Gompertz's equation calculated the inflection point and the maximal growth rate (Zwietering et al. 1990).

Biofilm formation

Biofilm biomass was measured on the plates that served for the 24 h toxicity test described above. Once the respiration scan was finished, the plates were thoroughly washed (five times) with PBS. Afterward, the biofilm biomass was quantified in crystal violet assay as Merritt et al. (2015) described. The reads (absorbance at $\lambda = 600$ nm) were recorded on BioTek Synergy H1 (BioTek Instruments, Winooski, VT, USA).

Statistical analysis

Statistical measures (mean, standard deviation) were counted using Origin 2021 software. The three-sigma (3σ) method was used to eliminate read errors that could arise because of the poor dispersion of nanomaterials. One-way ANOVA with Tukey's post hoc test was used for statistical comparisons between samples containing different concentrations of nanomaterials. Results with $p < 0.05$ were considered statistically different.

Results and discussion

Optical density and fluorescence

Optical density measurements suggested a variable response to the nanomaterial depending on the concentration. The comparisons of OD between samples are presented in Fig. 1. After 6 and 12 h, other reads have strengthened the results, providing more insight into population growth and inhibition dynamics. The nanomaterials did not lead to complete inhibition of bacterial growth, similar to the observation made in the previous studies (Sikora et al. 2018; Augustyniak et al. 2020b). All nanomaterials containing titanium dioxide or carbon nanotubes caused a decrease in the OD, especially in the highest concentrations.

Similarly, CoOF containing Co nanoparticles on the surface caused a significant decrease compared to the purified CoOF nanostructure. Each component that decreased OD in this study was also described as toxic against bacteria in the literature (Trukawka et al. 2019). On the other hand, carbon nanotubes added to the CoOF structure also could decrease the final OD in the cultures. It indicates that the seemingly biocompatible CoOF can be functionalized into a more toxic structure by adding CNT on its surface. Surprisingly, graphene oxide was characterized in this experiment as an OD increasing factor, suggesting a stimulative potential of this nanomaterial. This effect was powerful after 24 h. In the current literature, one can find evidence of the toxicity of graphene oxide against microorganisms and its growth enhancement potential, with the first option as the

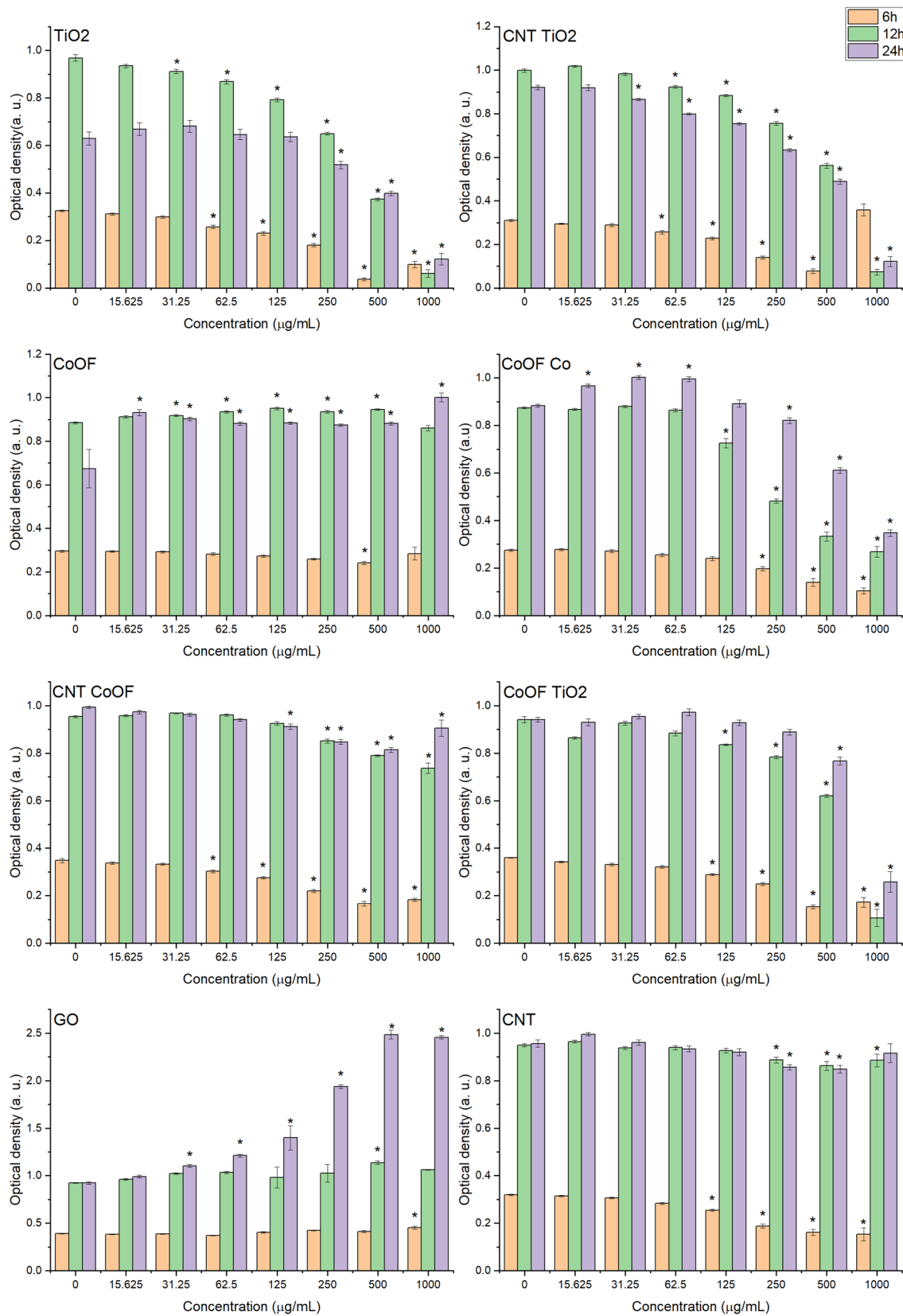


Fig. 1 The optical density of cultures after co-incubation with nanomaterials for 6, 12, and 24 h; *sample statistically different from the control sample (0) with $p < 0.05$

most frequent description (Yousefi et al. 2017; Zhang and Tremblay 2020). Considering also other “flake-like” structures considered in this study, it appears that these structures were rather biocompatible in the in vitro conditions created in our experiments. However, it should be emphasized that no additional dispersing factor was added, and these materials were tested in a liquid bacteriological medium. It could increase the agglomeration that lowered the overall toxicity, which was discussed as one of the common problems with studying nanomaterials on microbiological models (Ma et al. 2010; Augustyniak et al. 2021).

Selecting *P. aeruginosa* as a bacterial model for this study allowed the introduction the additional physiological measure, i.e., production of pigments (Palleroni 2015). These metabolites are considered virulence factors in pseudomonads, which overproduction should be avoided, particularly in therapeutic applications (Wang et al. 2016). Purified CoOF, CNT, and CNT/TiO₂ caused an increased fluorescence after 24 h in the middle range of tested concentrations and a drop in the highest quantities. Sole TiO₂ nanoparticles expressed an increase in fluorescence together with an increase in the nanomaterial concentration. On the other hand, the fluorescence in cultures containing CoOF/TiO₂, GO, and CoOF/Co decreased together with the increase in nanomaterial

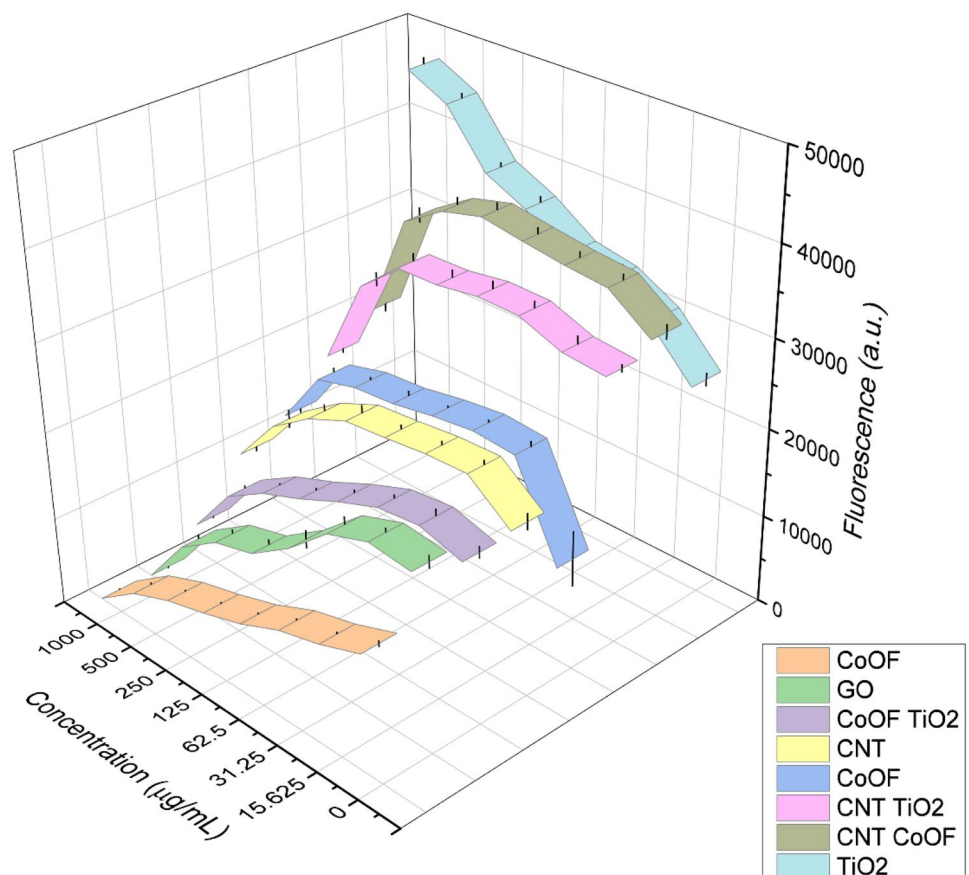
concentration. These data are shown in 3D Fig. 2 and separately in the Supplementary material (Fig. S6).

The results have indicated that in some concentrations, tested nanomaterials have a stimulative influence on pigment production in *P. aeruginosa*. This finding can be problematic from the perspective of medical applications, because it may suggest increased virulence (Trapet et al. 2016; Newman et al. 2017). On the other hand, these metabolites can find their use in biotechnology, e.g., as staining reagents for confocal microscopy (Brelje et al. 2002). Based on the obtained results, it can be assumed that including a range of nanomaterial concentrations in such studies may be proposed as a dynamic measure to assist safety assessment or stimulative potential of nanomaterial.

Respiration after 24 h incubation

The respiration measurements performed after 24 h incubation with nanomaterials have been previously conducted and published. However, these end-point tests included only a short incubation step and then a fluorescence reading (Sikora et al. 2018). In the current study, we have decided to scan the changes in the fluorescence during additional 4 h incubation with resazurin. This modification indicated the differences between the speed of transforming resazurin to resorufin,

Fig. 2 Fluorescence after 24 h cultures with the studied nanomaterials relating to the tested concentration



and showed differences in the shape of the resulting curve. At least two hypotheses can be formed trying to explain this phenomenon. The first one indicates that there might be some previously unknown changes in the bacterial metabolism that could affect the respiration rate. *P. aeruginosa* is known for its adaptability to environmental conditions, making this bacterium more resistant to toxicants, including antibiotics (Winstanley et al. 2016). In the second hypothesis, and seemingly more plausible in the scope of other results (e.g., OD and growth curves), the control might have expressed a lower respiration rate, because it had already reached the stationary phase. In this scenario, samples inhibited by nanomaterials reach the plateau phase later when the cells are still active after the logarithmic growth phase.

The current form of this assay provides more information than an end-point measurement. Furthermore, it has possibly revealed new phenomena that have not been observed before regarding the dynamics of cells' survival and activity in environments containing nanomaterials. One of the interesting examples investigated during this step was the response to GO. Considering the OD, all cultures containing the three highest GO concentrations (250–1000 µg/mL) should contain more biomass expected to transform resazurin faster. However, within these concentrations, only cultures containing 250 µg/mL and 500 µg/mL expressed higher activity characterized by a steep and fast fluorescence growth. The respiration was significantly impaired in the sample containing 1000 µg/mL (Fig. 3).

The changes in respiration dynamics in cultures may be an interesting research route to develop in further studies. Moreover, these observations may help biotechnological design use the studied nanomaterials (Nawrotek and Augustyniak 2015). Our results show that specific concentrations can generate the desired effect, including stimulating pigment production, higher biomass yield, or changes in respiration dynamics. It could be used in the biotechnological production of metabolites in the *P. aeruginosa* model. Nanomaterials have been suggested as stimulants in other microorganisms, such as *Shewanella oneidensis* (in flavin production) or *Streptomyces* sp. (in production of pigment and extracellular polymers) (Maurer-Jones et al. 2013a; Augustyniak et al. 2016).

Growth curves

The analysis of optical density results and respiration dynamics indicated that, in general, nanomaterials used in the concentration of 125 µg/mL could be considered as an effective dose for observable inhibition or stimulation (depending on the nanomaterial). Therefore, this concentration has been selected to perform a growth curve analysis.

This experiment allowed us to visualize the growth dynamics and assess them mathematically. This method is

commonly used in chemical engineering (Konopacki et al. 2020). As we have shown, it can also be applied to study the physiological response of *P. aeruginosa* to carbon nanocomposites and their components. This experiment has confirmed increased toxicity against tested bacterium in cultures containing TiO₂ and CoOF/Co (Fig. 4). These materials showed toxicity against bacteria in other studies (Lin et al. 2014; Trukawka et al. 2019). Here, the first one delayed the entering of the population into the logarithmic growth phase. However, once this occurred, the maximal growth rate (MGR) was even higher than in the control samples. CoOF/Co did not cause a significant delay in entering the logarithmic growth phase but considerably decreased MGR (Table 2). Nevertheless, the nanomaterial containing cobalt nanoparticles had the most potent antimicrobial effect on the surface. It could be attributed to the known toxicity of cobalt nanoparticles, which was previously described on reference strains of *P. aeruginosa* and *S. aureus* (Trukawka et al. 2019). Interestingly, another version of this material (CoOF) containing cobalt but trapped between the flakes did not cause growth inhibition. Furthermore, a stimulative potential of nanomaterials was revealed in this test, particularly with the flake-like nanomaterials (i.e., purified CoOF and GO). At the end of the experiment, these structures reached the highest OD, which agrees with the literature, where a growth stimulation by graphene was described (Zhang and Tremblay 2020). Another nanomaterial containing titanium dioxide (CNT TiO₂) did not delay the exponential growth, although it significantly slowed down MGR.

*K+ is the mean value for controls used in the study

Biofilm formation

The biofilm formation assay indicated a variable response to tested nanomaterials (Fig. 5). *P. aeruginosa* is a potent biofilm producer (Milivojevic et al. 2018). Biofilming in pseudomonads is significant in therapy, because the biofilm structure provides the cells with additional protection from environmental stresses, making them more difficult to eliminate (Gambino and Cappitelli 2016).

In general, the outcome depended on nanomaterial concentration. The general tendencies in response to tested nanomaterials are shown in Fig. 5. These results can also be viewed in greater detail in the Supplementary materials (Fig. S7). Even though the middle range of concentrations could cause a decrease in biofilming in the cultures (particularly CNT/TiO₂, GO, and CoOF), reads recorded in samples containing the highest amounts of nanomaterials were increased. It could be caused by trapping the nanomaterial in the biofilm matrix, making nanomaterials a sort of nucleation centers (Flemming et al. 2016). The observed drop in the middle range of tested concentrations could be caused by the increased aggregation of cells around the

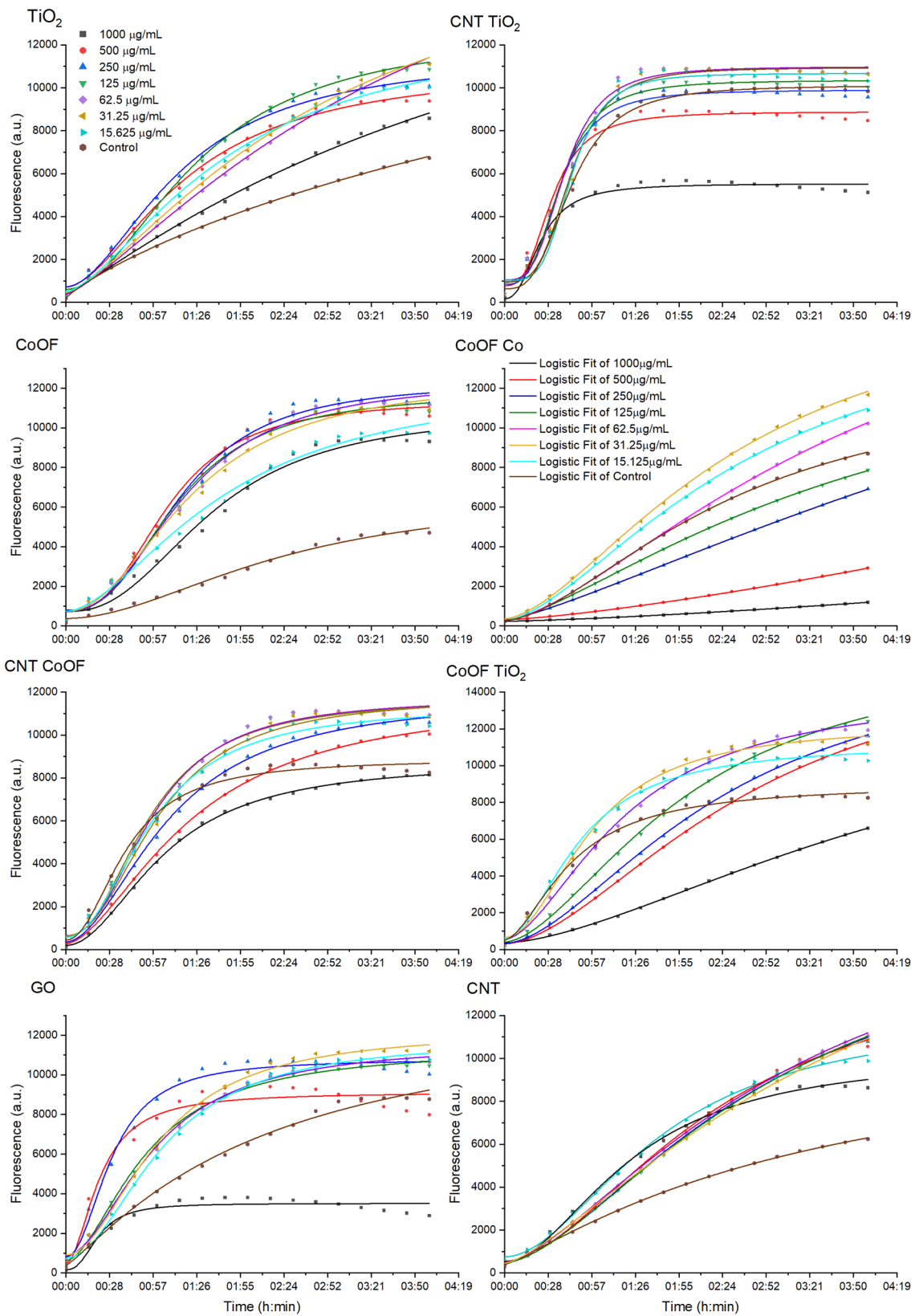


Fig. 3 Respiration of *P. aeruginosa* cells after 24 h incubation with studied nanomaterials; each panel is dedicated to another nanomaterial, presented legends apply to all panels

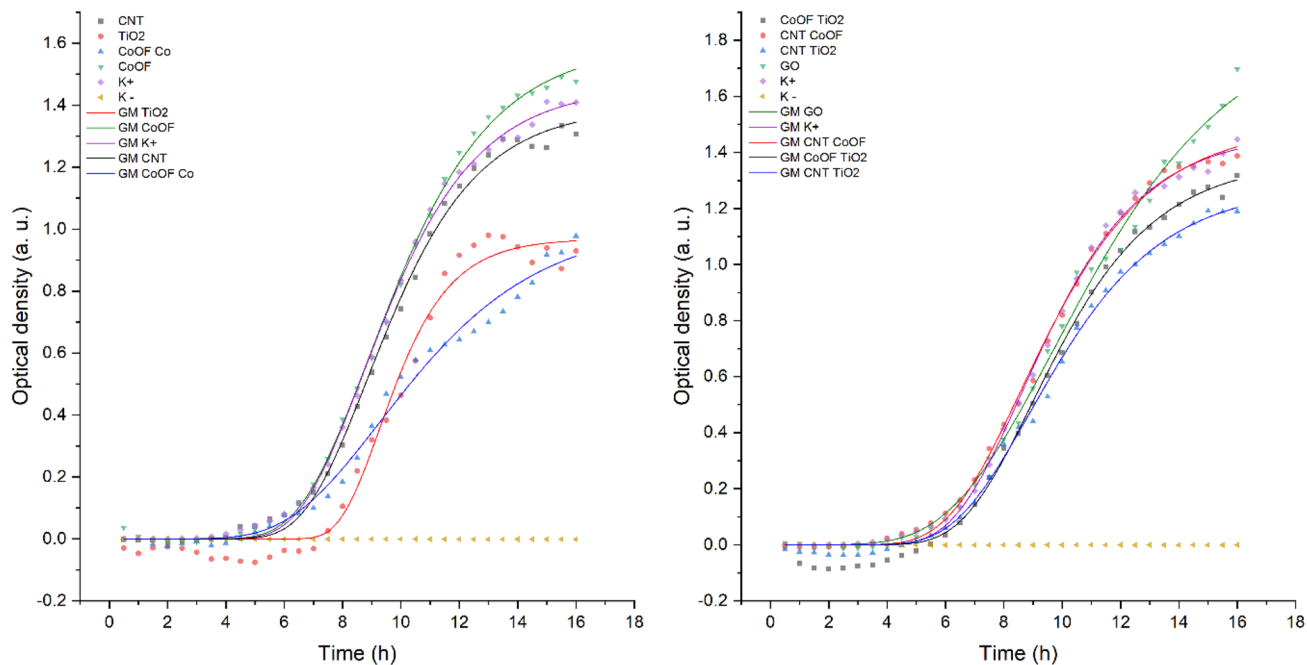


Fig. 4 Growth curves of *P. aeruginosa* population cultivated with studied nanomaterials

Table 2 Inflection point and maximal growth rate of *P. aeruginosa* cultured with studied nanomaterials, in comparison to control samples

Nanomaterial	Inflection point [h]	Max growth rate [h^{-1}]
CNT	8.83	0.24
TiO ₂	9.27	0.26
CoOF/Co	9.28	0.12
CoOF	8.91	0.25
CoOF/TiO ₂	8.97	0.21
CNT/CoOF	8.58	0.22
CNT/TiO ₂	8.89	0.18
GO	9.72	0.20
K+*	8.67	0.24

nanomaterial. However, if these agglomerates have not been firmly attached to the forming biofilm, they could be washed out during the staining procedure (Merritt et al. 2015). The agglomeration of pseudomonads promoted by nanomaterials has been previously described for carbon nanotubes and silica nanotubes (Ma et al. 2010; Augustyniak et al. 2020a). In the current studies, signs of such aggregation can be found in Fig. 4, in the population's growth curve co-incubated with TiO₂. It was the probable cause for the delay in entering the logarithmic growth phase, similarly to phenomena described for silica nanotubes (Augustyniak et al. 2020a).

Conclusions

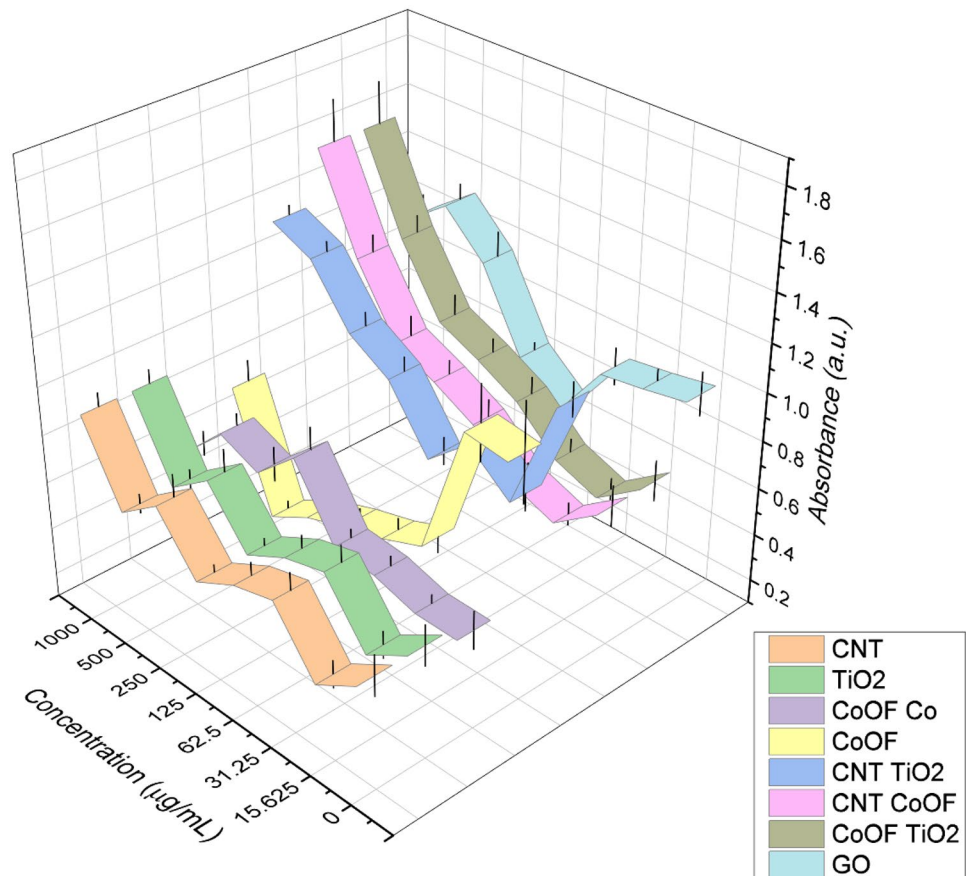
Pseudomonas aeruginosa can be considered an interesting bacterial model for investigating the activity of nanomaterials because of its adaptability and capability to produce metabolites that can serve as physiological state indicators (pigments).

The results have confirmed that the type and concentration of nanomaterial can influence the physiological features of this microorganism. Furthermore, the structure of the nanomaterial, its components, and concentration may specifically induce or inhibit physiological characteristics such as the secretion of fluorescent pigments or biofilm formation. These observations can be further studied to verify whether nanomaterials in specific concentrations can be used to develop stress-induced production of desired secondary metabolites (e.g., rhamnolipids or pyocyanin).

The experimental approach allowed finding sublethal concentrations of selected nanomaterials (mainly at the level of 125 $\mu\text{g}/\text{mL}$) and the concentrations that can stimulate population growth. This finding could be helpful in the biomass production process if the cultures tested in our study can be scaled up. Furthermore, introducing additional points in OD measurements (particularly the additional measurement after 6 h) helped record inhibiting doses of nanomaterials and could be introduced to the standard practice, which was verified with performing the growth curve analysis.

Among the studied cases, nanomaterials displaying cobalt nanoparticles on the surface or TiO₂ nanoparticles showed

Fig. 5 Biofilm formed by *P. aeruginosa* in response to studied nanomaterials in various concentrations



the most profound antimicrobial effects. Nevertheless, even though it might have been inhibited, the bacterial population still could develop a considerable amount of biomass.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13204-022-02460-3>.

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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