Volume 12
Number 6
June 2025
Pages 2889–3382

Environmental Science Nano

rsc.li/es-nano

ISSN 2051-8153

ROYAL SOCIETY OF CHEMISTRY

PAPER

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ROYAL SOCIETY OF CHEMISTRY

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Cite this: *Environ. Sci.: Nano*, 2025, 12, 3018

Ecotoxicological assessment of MWCNT-reinforced MOC composites: impacts on model bacteria and eukaryotes with environmental relevance†

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Composite materials based on magnesium oxychloride cement (MOC), reinforced with multi-walled carbon nanotubes (MWCNT) and their oxidized form (MWCNT-ox), are promising eco-friendly building materials. However, little is known about their ecotoxicological impact. This study pioneers the evaluation of MWCNT-reinforced MOC effect on selected prokaryotic (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*) and eukaryotic (*Artemia salina*, *Sinapis alba*, *Desmodesmus subspicatus*) organisms. Initially, the effects of MWCNT and MWCNT-ox at concentrations of 1 g L^{-1}, 0.1 g L^{-1}, and 0.01 g L^{-1} on the growth and proliferation of organisms were assessed. While MWCNT samples did not affect bacterial growth or eukaryotic viability, they significantly inhibited bacterial biofilm formation. The antibiofilm effect varied among the tested bacteria, with *S. aureus* and *E. coli* being significantly more inhibited than *P. aeruginosa*. No differences were observed between the effects of MWCNT and MWCNT-ox on bacteria, while MWCNT-ox exhibited higher toxicity toward the tested eukaryotic organisms. Subsequently, MOC reference (MOC-REF) and MWCNT-reinforced MOC samples (MOC-MWCNT, MOC-MWCNT-ox) were prepared and characterized using XRD and SEM-EDS. Ecotoxicological assays confirmed that the composites inhibited both bacterial growth and biofilm formation, a highly desirable outcome, as microbial degradation compromises the longevity of building materials. The incorporation of MWCNT enhanced the antibacterial effect of MOC. Further, the addition of MWCNT and MWCNT-ox to MOC did not affect *A. salina* mortality, *S. alba* seed germination, or *D. subspitatus* growth. However, inhibition of *S. alba* root growth was observed at the highest tested concentration (1 g L^{-1}) for all MOC samples, regardless of MWCNT presence. Overall, the results indicate a low environmental impact of the prepared MOC-MWCNT and MOC-MWCNT-ox composites.

Received 20th November 2024,
Accepted 20th May 2025

DOI: 10.1039/d4en01088d

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Environmental significance

Composite materials based on magnesium oxychloride cement (MOC) are promising eco-friendly building materials. Their physical and mechanical properties can be significantly enhanced by the incorporation of nanoadditives such as multi-walled carbon nanotubes (MWCNT) and their oxidized form (MWCNT-ox). However, their environmental impact remains largely unexplored. This study systematically investigates the effects of MWCNT, MWCNT-ox, and MOC reinforced by them on prokaryotic and eukaryotic organisms. The research provides fundamental insights into the interactions between these materials and biological systems, including their influence on bacterial viability and biofilm formation, and eukaryotic mortality, germination, and growth. The findings indicate that the tested materials have a low environmental impact, addressing emerging concerns regarding their potential ecotoxicity, and may accelerate their applications.

1. Introduction

Composites based on magnesium oxychloride cement (MOC) are an eco-friendly alternative to Portland cement- (PC-) based construction composites.[1] These composites are based on the MOC phase 5 matrix ($Mg_3(OH)_5Cl{\cdot}4H_2O$), which provides high strength, high abrasion resistance, low thermal conductivity, and fire resistance.[2–4] The main advantage of MOC over PC is its increased environmental sustainability gained through the

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† Electronic supplementary information (ESI) available: Verification of MOC-REF, MOC-MWCNT, and MOC-MWCNT-ox sterility (Fig. S1); *P. aeruginosa* suspension after the incubation with MOC materials (Fig. S2). See DOI: https://doi.org/10.1039/d4en01088d

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lower energy demands on the production of its raw materials and its increased ability to sequester carbon dioxide from the atmosphere.[5–7] Current research on MOC-based composites mainly addresses their primary drawback, poor water resistance.[8] Researchers generally take the approach of modification of the composites through the application of specific substances based on the additive-accommodation ability of MOC. In this regard, various types of inorganic and organic modifiers were tested, showing their efficiency as water-induced damage retardants.[9–13] Among these MOC-based materials, carbon-based nanomaterials have shown significant promise. The used carbon-based materials can be divided into the layered (2D) and 1D groups. It was shown that the layered nanomaterials, such as graphene or graphene oxide, are beneficial mostly in the tuning of microstructural parameters, which directly influence the mechanical and hygric parameters.[14] From the group of 1D carbon nanomaterials, carbon nanotubes (CNT) and their oxidized forms (CNT-ox) are among the most researched ones.

The incorporation of CNT into construction composites is an experimental approach yielding positive results in terms of material properties. This is attributed to the outstanding mechanical, hygric, thermal, and electrical characteristics of CNTs.[15] Furthermore, these carbon nanoadditives can help heal cracks by forming bridges, filling pores, and promoting hydration of the cementitious phases in the matrix.[16] Utilization of CNT in MOC-based composites mainly focuses on the use of multi-walled carbon nanotubes (MWCNT) and oxidized multi-walled carbon nanotubes (MWCNT-ox). Studies have shown that MOC-MWCNT composites exhibit increased compressive strength (higher than 70 MPa), stiffness, and, importantly, improved water resistance (softening coefficient higher than 60, water absorption coefficient lower than 0.001 kg m^{-2} $s^{-1/2}$), even with low MWCNT or MWCNT-ox concentrations (0.02–1.0 wt% relative to the weight of the matrix).[17–19] While both MWCNT and MWCNT-ox improve the MOC material quite similarly, MWCNT-ox has a more pronounced effect on flexural strength due to the present oxygen functionalities, resulting in flexural strength values around 20 MPa.[20]

Given the promising industrial applications of MOC-MWCNT materials, their ecotoxicity has become a significant concern. Regarding MWCNT, studies on their toxicity in various organisms—microorganisms, plants, algae, crustaceans, and fish—have yielded inconsistent results.[21] MWCNTs can either inhibit or promote microbial growth, depending on the microbial species, MWCNT type, concentration, structure (*e.g.*, particle diameter, surface area), and environmental conditions.[21–25] It is hypothesised that the primary mechanisms proposed for MWCNT microbial toxicity include: (i) production of reactive oxygen species (ROS),[26] (ii) direct damage to bacterial cell membranes, and (iii) chemical–physical interactions between MWCNT and microbial cells.[27] Reduced MWCNT toxicity may be attributed to CNT encapsulation by organic matter, limiting MWCNT mobility.[28] In contrast, MWCNTs can promote microbial growth by inducing specific gene expression that accelerates cell division.[29]

For eukaryotic organisms, the effects of MWCNT are similarly varied, with both toxic and beneficial outcomes reported. Specific conclusions are difficult to draw as data on certain organisms remain limited.[24] For example, a study by Yang, Deng[30] on the plant *Arabidopsis thaliana* showed that MWCNT exposure can affect root growth and induce stress responses in plant cells, while another study reported enhanced photosynthesis, protein expression, and lateral root growth.[24] In some cases, exposure to MWCNTs led to increased ROS accumulation and membrane disruption, inhibiting plant growth and physiological functions, as observed in lettuce *Brassica rapa*.[31]

In aquatic organisms, sublethal effects like altered swimming behavior and feeding rates were reported for the crustacean *Daphnia magna* following MWCNT exposure.[32] Similarly, studies on fish (*Danio rerio* and *Cyprinus carpio*) demonstrated that MWCNTs can cause developmental abnormalities, increased embryo mortality (suggesting adverse effect on reproductive and developmental processes), oxidative stress, and tissue damage, specifically gill and liver.[33,34]

The diversity of MWCNT effects on organisms raises questions about their toxicity when incorporated into composite materials like MOC. While data is limited, as with MWCNT alone, the conclusions are inconsistent. Some studies have shown that MWCNTs combined with other materials, such as ZnO–Ag nanocomposites, enhance antibacterial activity against *E. coli* and *S. aureus*.[23] Regarding eukaryotes, co-exposure of MWCNT with heavy metals[35,36] or organic pollutants, such as phenanthrene,[37] has been shown to increase toxicity and bioaccumulation in *Daphnia magna*. Negative effects of combining MWCNT with nonylphenol compared to the pure substance have also been demonstrated in the earthworm *Eisenia fetida*.[38] In contrast, a reduction in toxicity was observed when MWCNTs were combined with bromodiphenyl ether in a study conducted on *D. rerio*.[39] Additionally, exposure to MWCNT enhanced the growth of broccoli seeds subjected to salt stress.[40]

Despite the increasing research into MOC composites and the promising effects of their modification with MWCNTs, the ecotoxicological impact of MWCNT-reinforced MOC has not been systematically investigated yet. This study aims to fill that gap by evaluating the effects of two types of MWCNTs and MWCNT-modified MOC on the growth and proliferation of three prokaryotic (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*) and three eukaryotic (*Artemia salina*, *Sinapis alba*, *Desmodesmus subspicatus*) organisms. Model organisms were selected to represent those commonly used in ecotoxicology and those relevant to microbial degradation and human health risks.

2. Materials and methods

2.1 Raw materials

The MgO powder (purity 98%) was sourced from penta, s.r.o., Prague, Czech Republic. $MgCl_2 \cdot 6H_2O$ (p.a. purity) was obtained from Lach-ner, s.r.o., Neratovice, Czech Republic.

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Three different size fractions of silica sand—PG1 (0.0–0.5 mm), PG2 (0.5–1.0 mm), and PG3 (1.0–2.0 mm)—were provided by Filtrační písky Ltd., Chlum u Doks, Czech Republic. MWCNT and MWCNT-ox, both with a purity >95%, were purchased from Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences (Chengdu, China). Purchased nano-dopants, MWCNT and MWCNT-ox, were studied using TEM and SEM (detailed information can be found in a previous publication[41]). MWCNT and MWCNT-ox were dissolved in sterile tryptone soy broth (TSB, Oxoid Ltd., United Kingdom) in concentrations of 1 g L^{-1}, 0.1 g L^{-1}, and 0.01 g L^{-1}, and used for the ecotoxicological assay.

2.2 MOC samples preparation and characterization

A reference material, MOC-REF, was prepared without MWCNT and MWCNT-ox for comparison purposes. Both composite samples, MOC-MWCNT and MOC-MWCNT-ox, contained their respective nano-dopants at a concentration of 1.0 wt% relative to the weight of the cement paste. Table 1 presents the composition of prepared MOC composites. To prepare the MOC samples, $MgCl_2{\cdot}6H_2O$ was dissolved and combined with MgO powder and silica sand using a mixer. For the MOC-based composites, the respective nano-dopant was homogenized in $MgCl_2{\cdot}6H_2O$ solution and mixed with MgO and silica sand. The wet mixtures were poured into prismatic molds. The samples were demolded after one day, and then left to cure in the air for 27 days. A more detailed preparation procedure is described in previous publications.[18] The prepared composite samples were studied using XRD and SEM-EDS; the instrumentation information and settings can be found in previously published publications.[41,42]

2.3 Verification of MOC materials sterility

MOC composites both in solid and powder form were incubated in (i) sterile TSB for 48 h at 30 °C and (ii) plate count agar (PCA, Merck, Germany), respectively, for 72 h at 30 °C. After cultivation, the absorbance of the TSB was measured spectrophotometrically at 620 nm (Tecan, Switzerland) and compared to the values determined before cultivation, and colony-forming units (CFU) formed on PCA were counted.

2.4 Bacterial strains, eukaryotic organisms, and culture conditions

Three bacterial strains (international standard reference strains for antibacterial disc susceptibility testing) obtained from the Czech Collection of Microorganisms (CCM, Czech Republic) were used as model microorganisms in this study: Gram-positive bacterium *Staphylococcus aureus* ATCC 25923 (eq. CCM 3953) and Gram-negative bacteria *Escherichia coli* ATCC 25922 (eq. CCM 3954) and *Pseudomonas aeruginosa* ATCC 27853 (eq. CCM 3955). Pure bacterial cultures, statically cultivated in sterile TSB for 24 h at 37 °C, were used for the ecotoxicological assays described below. Further, three eukaryotic organisms were included in testing: *Artemia salina* (aquatic crustacean; Easyfish, Czech Republic), *Sinapis alba* (mustard; Biom, Czech Republic), and *Desmodesmus subspicatus* (algae; Institute of Botany Třeboň, Czech Republic).

2.5 Ecotoxicological assay – MWCNT and MWCNT-ox

2.5.1 Impact of MWCNT on bacterial growth and biofilm formation. First, bacterial growth screening in the presence of MWCNT or MWCNT-ox in three concentrations (1 g L^{-1}, 0.1 g L^{-1}, 0.01 g L^{-1}) was performed using a spectrophotometer (BioTek Synergy H1, Agilent, US). These concentrations were selected as a standard gradient for testing various substances and materials, enabling comparisons across studies. Absorbance (λ = 600 nm) of mixtures composed of TSB and MWCNT/MWCNT-ox was measured continuously at 37 °C for 24 h under continuous orbital mixing. After subtracting blank values, growth curves were constructed and maximal specific growth speed was determined.

Next, the impact of tested MWCNTs on bacterial biofilm formation was investigated. Biofilm was formed in a 96-well microtiter plate (Gama Group, Czech Republic) under the following conditions. Each well contained 160 μL of sterile TSB, 20 μL of single-species bacterial suspension with optical density 0.5 MacFarland (McF), and 20 μL of MWCNT (ev. MWCNT-ox) solution containing TSB to ensure the final concentration of MWCNT 1 g L^{-1}, 0.1 g L^{-1}, and 0.01 g L^{-1}, respectively, in a well. Controls of microbial growth contained 180 μL of TSB and 20 μL of single-species bacterial suspension with optical density 0.5 McF; controls of MWCNT media contained 180 μL of TSB and 20 μL of MWCNT-containing TSB. Plates were incubated at 37 °C for 24 h. After cultivation, biofilms were washed five times with saline solution, dried for 45 min at laboratory temperature, and stained with 0.1% crystal violet (CV, Sigma-Aldrich, US) for 45 min at laboratory temperature. After washing three times with saline solution, the samples were either (i) microscope under optical microscopy at the 60× magnification or (ii) incubated with 200 μL of 96% ethanol for 15 min at laboratory temperature, from which 100 μL was transferred into a sterile 96-well microtiter plate and the A_{595nm} of the suspensions was measured

Table 1 Mixture composition of MOC-REF, MOC-MWCNT, MOC-MWCNT-ox (g)

Sample	MgO	$MgCl_2{\cdot}6H_2O$	H_2O	MWCNT	MWCNT-ox	PG1	PG2	PG3
MOC-REF	106.2	107.2	66.5	—	—	106.3	106.3	106.3
MOC-MWCNT	106.2	107.2	66.5	2.8	—	106.3	106.3	106.3
MOC-MWCNT-ox	106.2	107.2	66.5	—	2.8	106.3	106.3	106.3

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spectrophotometrically (Tecan, Switzerland). Further, we tested the effect of MWCNTs on the formation of ROS; similar conditions as in previous microbial tests were used. H_2O_2 (oxidative stress marker) production was quantified using the ROS-Glo™ H_2O_2 Assay (Promega, US) according to the manufacturers protocol, with final measurement of luminescence (BioTek Synergy H1, Agilent, US) and determination of percentage increase of ROS generation compared to the control (bacteria without MWCNT addition).

2.5.2 Determination of MWCNT toxicity on eukaryotic organisms. The mortality test with *Artemia salina* was done according to standard laboratory procedure with modifications. At first, *Artemia* cysts were hatched in saltwater for 24 h at 26 °C with the use of a hatching device (JBL, Germany) and continuous illumination. Then, the nauplii were transferred to a demineralized water with 30 g L^{-1} of sodium chloride. Samples were prepared by dissolving the nanomaterial powder in a control solution consisting of demineralized water and 30 g L^{-1} of sodium chloride. After the sonication (ultrasonic bath, 30 min), different concentrations (1 g L^{-1}, 0.1 g L^{-1}, 0.01 g L^{-1}) by dilution with 30 g L^{-1} sodium chloride solution were immediately prepared. As a standard solution, potassium dichromate dissolved in a control solution was used. Hatched nauplii were transferred to a Petri dish (10 pcs per 1 dish), and 10 mL of diluted samples, control, or standard solution was added. Prepared dishes were put in an incubator with a temperature set to 20 °C and kept in the dark. Experiments were performed in triplicate. After 24 hours, the dead individuals were counted, and mortality was evaluated. The potential for bioaccumulation was identified through microscopic examination (Primo Star, Zeiss, Germany) with 40× magnification.

The growth of mustard *Sinapis alba* was tested according to a standard procedure with modifications. Prior to testing, the samples were prepared by dispersion of nanopowder in a medium and left for sonication for 30 min. Then, the series of dilutions 1 g L^{-1}, 0.1 g L^{-1}, and 0.01 g L^{-1} was prepared by dilution with standard control medium. A control was established using demineralized water enriched with salt solution. Additionally, a solution of potassium dichromate was used as a reference substance in the positive control. The experiments were carried out in 120 mm diameter Petri dishes comprising cellulose filter papers with holes, with a total volume of 5 mL of the prepared samples or control added to each dish. A total of fifteen seeds were placed into the holes, after which each dish was covered with a lid. Subsequently, the samples were incubated at 20 °C for a period of 72 h in the absence of light. Following the incubation period, root length was quantified utilizing ImageJ software (National Institutes of Health, United States). A seed was deemed germinated when a root of at least 1 mm was observable. The germination of seeds was enumerated, and root elongation inhibition was calculated by comparison with the control results.

The algal growth inhibition of *Desmodesmus subspicatus* was conducted in accordance with the standard EN ISO 8692, with the incorporation of modifications. The green algae were obtained as a sterile culture of *D. subspicatus* (R. Chodat), E. Hegewald et A. Schmidt, Brinkmann 1953/SAG 8681. Three days prior to the inhibition test, the algae were subjected to a pre-cultivation process in a 500 mL Erlenmeyer flask, which was filled with Bold's Basal growth medium (BBM) and sealed with a pulp plug. The incubation was on an orbital shaker (ELMI DOS-20L, ELMI USA) at 23 °C under continuous illumination provided by daylight lamps, with an intensity of 7000 lx. The samples were diluted with BBM medium and subjected to ultrasonication for a period of 30 min. The final concentration was 0.01 g L^{-1} and 0.1 g L^{-1}, as a precaution against potential shading effects. Thereafter, the samples were transported under sterile conditions to Erlenmeyer flasks with a total volume of 15 mL, together with the algal inoculum. The volume of added algal inoculum was dependent on the initial density determined under microscopy (Primo Star, Zeiss, Germany) with 400× magnification and Bürker counting chamber. The flasks were cultivated under identical heat and light conditions to those employed during the pre-cultivation phase, which was conducted under orbital shaking (150 rpm). BBM served as a control medium. Following a period of 72 h, the cell concentration of *D. subspicatus* was quantified utilising a microscope. The resulting growth and inhibition rates were calculated according to the standard.

2.6 Ecotoxicological assay – MOC samples

Firstly, the impact of MOC samples on bacterial growth was studied. Pieces (1 × 1 × 1 cm) of MOC samples, respectively, were incubated in 3 mL of single-species bacterial suspensions of *E. coli*, *S. aureus*, and *P. aeruginosa* of optical density 0.5 McF at 37 °C for 48 h. Controls of bacterial growth contained only 3 mL of bacterial suspension; control of MOC sterility contained 3 mL of sterile TSB and an MOC piece. Both before and after the cultivation, $A_{620\ nm}$ of the suspension was measured spectrophotometrically (Tecan, Switzerland). Further, the pH of suspensions both before and after cultivation was measured with a calibrated pH meter (Radiometer Analytical, France).

In the next step, the impact of MOC samples on bacterial biofilm formation was determined. Pieces (1 × 1 × 1 cm) of MOC samples, respectively, were incubated in 3 mL of single-species bacterial suspensions of *E. coli*, *S. aureus*, and *P. aeruginosa* of optical density 0.5 McF at 37 °C for 48 h. Controls of bacterial growth contained only 3 mL of bacterial suspension; control of MOC sterility contained 3 mL of sterile TSB and an MOC piece. After the cultivation, MOC samples were washed in saline solution and sonicated in 3 mL of sterile saline solution for 3 min at room temperature in a sonication bath (Polsonic, Poland). Physiological solution with released biofilm-forming cells was decimally diluted in sterile saline solution up to the eighth dilution. Droplets (20 µl) of all dilutions were plotted on PCA (Merck, Germany) in triplicates and

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cultivated. After the cultivation (24 h at 37 °C), CFUs were counted, values were converted to 1 cm^2, and compared to the control. Furthermore, SEM analysis of bacteria adhered to the MOC surface was performed, along with EDS for confirmation of organic matter presence according to the previously published publications.[41–43] Further, H_2O_2 production in MOC presence was quantified using the ROS-Glo™ H_2O_2 Assay (Promega, US) similarly as described in the section 2.5.1.

Finally, determination of MOC-MWCNT toxicity on eukaryotic organisms was performed. *A. salina* mortality, *S. alba* growth, and *D. subspicatus* growth in the presence of MOC samples, respectively, was determined similarly as described in 2.5.

2.7 Statistical analysis

All the experiments were performed at least in biological and technical triplicates. Data analysis was provided in the R programming language. Outliers were discarded according to Dean-Dixon's *Q*-test. The Shapiro–Wilk test was applied to data sets to verify normal distribution; the data were considered normally distributed at $p > 0.05$. The final data were subjected to multiple comparisons by one-way analysis of variance (ANOVA) with a significance level $\alpha = 0.05$, $\alpha = 0.01$, and $\alpha = 0.001$. In case of significant results, Tukey HSD (Tukey honest significant differences) and Bonferroni correlation were applied to perform multiple pairwise comparisons of groups' mean.

Statistical analysis was performed for the evaluation of the initial hypotheses. We presumed that both tested forms of MWCNT, as well as the prepared MOC samples, influence bacterial and eukaryotic growth and viability, and the different effects of MWCNT and MWCNT-ox (eventually MOC-MWCNT and MOC-MWCNT-ox) were expected.

3. Results and discussion

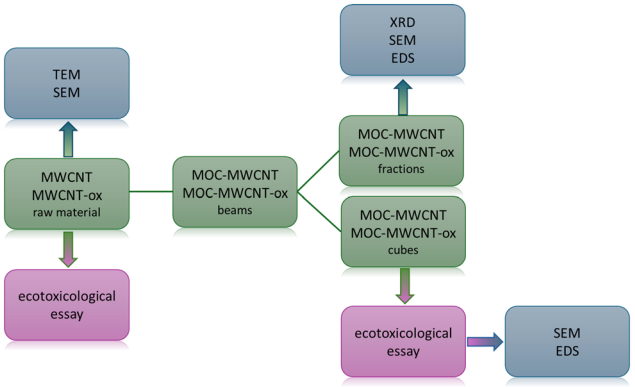
For better clarity and understanding, a visualization of sample preparation and characterization can be seen in Scheme 1.

3.1 MWCNT and MWCNT-ox characterization

The purchased nano-dopants, MWCNT and MWCNT-ox, were characterized using TEM and SEM (Fig. 1). Both nano-dopants exhibited the typical morphology of carbon nanotubes, appearing as disorganized, long carbon tubes with an approximate diameter of 25 nm. No significant difference between MWCNT and MWCNT-ox was observed. SEM-EDS analysis confirmed that oxygen content in MWCNT-ox was significantly higher compared to MWCNT.

3.2 Impact of MWCNT on prokaryotes and eukaryotes

First, the impact of MWCNT and MWCNT-ox on bacterial growth was examined. Spectrophotometric measurement (Fig. 2) showed that the presence of MWCNT and MWCNT-ox



Scheme 1 Visualization of sample preparation and characterization.

at all tested concentrations (1 g L^{-1}, 0.1 g L^{-1}, 0.01 g L^{-1}) did not significantly affect the growth of any of the tested bacteria ($\alpha = 0.05$). This is probably caused by the aggregation and settling of MWCNT and MWCNT-ox particles. Growth curves and quantitative growth characteristics, such as the maximum specific growth rate, did not differ significantly across the tested bacteria.

Our data are in correspondence with recent studies,[21,44] also concluding that MWCNT do not necessarily influence bacterial growth. MWCNTs are generally considered less toxic than single-walled carbon nanotubes (SWCNTs), and their effect can vary depending on several factors, such as particle size, concentration, dispersion quality, functionalization, and the type of target bacteria.[22,23] However, studies reporting that MWCNT inhibit bacterial growth prevail, particularly at higher concentrations of MWCNT. For example, Saleemi, Fouladi[25] reported an antibacterial effect of MWCNT at

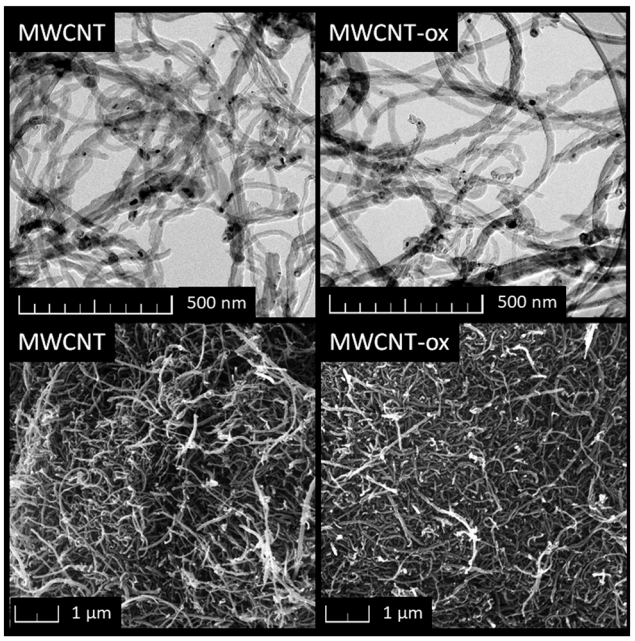


Fig. 1 TEM (top) and SEM (bottom) micrographs of MWCNT and MWCNT-ox.

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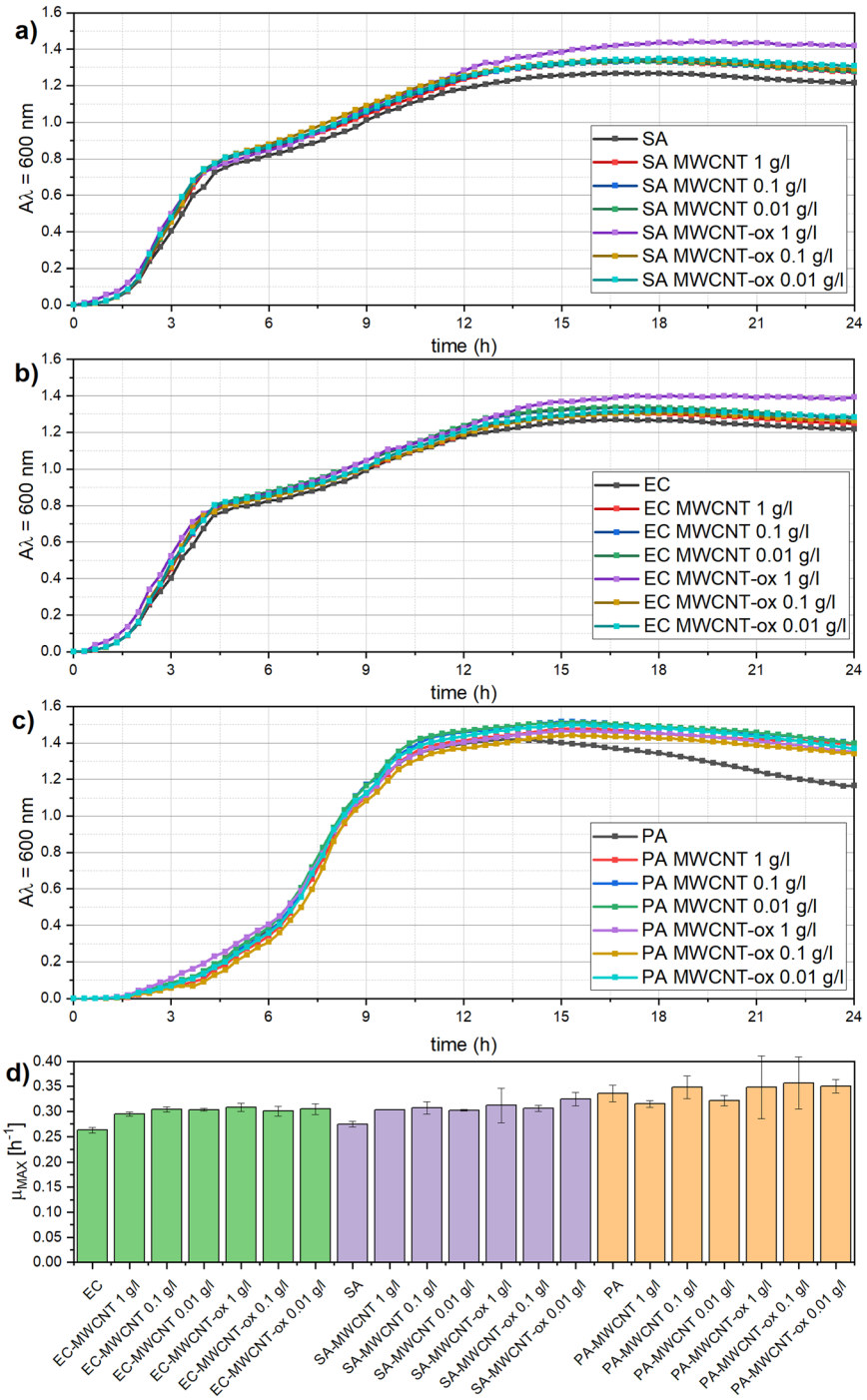

Fig. 2 Influence of MWCNT and MWCNT-ox on bacterial growth; a–c: growth curves of *S. aureus* (a), *E. coli* (b), and *P. aeruginosa* (c); d: maximum growth rate (exponential phase fitted with a linear line, its slope is the maximum growth rate) of *E. coli* (EC), *S. aureus* (SA) and *P. aeruginosa* (PA) in the presence of different concentrations (1 mg mL^{-1}, 100 μg mL^{-1}, 10 μg L^{-1}) of MWCNT and MWCNT-ox, respectively.

concentrations between 60 and 100 μg mL^{-1}, while lower concentrations had no significant impact. In the study of Liu, Wei,[22] a high dose of 500 μg mL^{-1} significantly inhibited the growth of *E. coli* and *S. aureus*. Given the variability in MWCNTs' effects, it is critical to test each type of MWCNT before application due to the complexity and inconsistency of its inhibition potential.

In contrast, both MWCNT and MWCNT-ox exhibited strong antibiofilm activity at all tested concentrations (1 g L^{-1}, 0.1 g L^{-1}, 0.01 g L^{-1}) against the three bacterial species (Fig. 3). Biofilm formation in the presence of both MWCNT and MWCNT-ox was significantly reduced compared to the control ($p < 0.05$). However, the inhibition effect varied between the bacteria, with *S. aureus* and *E. coli* being

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significantly more inhibited than *P. aeruginosa* ($p < 0.001$). No significant differences were observed between MWCNT and MWCNT-ox in their effect on *S. aureus* and *E. coli* across all concentrations. However, *P. aeruginosa* was more strongly inhibited at a concentration of 0.1 g L^{-1} for both MWCNT and MWCNT-ox. The differential effects observed on the bacterial species are likely due to differences in cell wall structure and shape between Gram-positive and Gram-negative bacteria, which are considered the main factors influencing bacterial resilience to external factors.[45–47]

Our results demonstrate that while MWCNT and MWCNT-ox do not inhibit bacterial growth, they exert strong antibiofilm effects. This could be due to the settling of MWCNT particles at the bottom of the culture plate, preventing bacterial adhesion and continuous biofilm formation. Additionally, MWCNTs are thought to induce oxidative stress and generate ROS, which hinder biofilm formation.[26] In the literature, antibiofilm effects of MWCNT themselves have been studied rarely, as they are more often tested in composites that enhance this effect. For instance, Gomes, Gomes[46] observed strong antibiofilm activity of MWCNT/poly(dimethylsiloxane) composites against *E. coli* and *Enterococcus faecalis*, with increasing MWCNT content correlating with stronger antibiofilm activity. Similarly, Al-Gaashani, Pasha[23] reported antibiofilm activity inducing effect of MWCNT in ZnO-Ag-MWCNTs nanocomposites against *E. coli* and *S. aureus*.

Prokaryotes and eukaryotes exhibit fundamental physiological differences, which result in varied responses to environmental factors. In addition to the bacterial tests

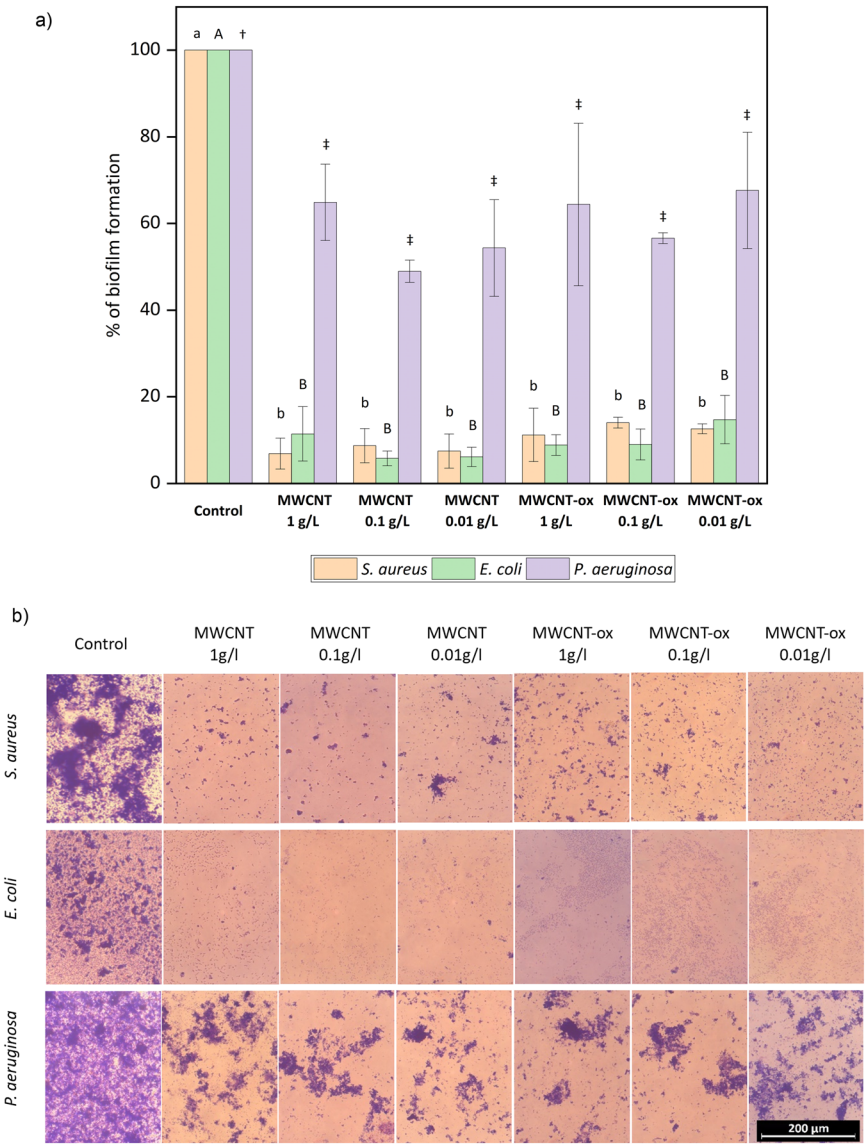

Fig. 3 Influence of MWCNT and MWCNT-ox (1 g L^{-1}, 0.1 g L^{-1}, 0.01 g L^{-1}) on bacterial biofilm formation: a) quantitative analysis using CV staining (means that do not share a letter within the same column are statistically significant at $p < 0.05$), b) qualitative analysis using CV staining and optical microscopy.

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mentioned above, the mortality rate of *A. salina*, germination and root growth inhibition in *S. alba*, and growth inhibition of *D. subspicatus* were evaluated.

To investigate the effects of oxidized and non-oxidized MWCNT on crustaceans, *A. salina* nauplii were used. This early life stage is generally considered more sensitive than the adult form. No significant mortality or bioaccumulation was observed for MWCNT and MWCNT-ox at concentrations of 0.01 and 0.1 g L^{-1} (Table 2). A slight increase in mortality was observed at 0.1 g L^{-1} for MWCNT-ox, possibly due to its lower potential for aggregation and thus higher bioavailability.[48] These findings are consistent with studies of Mesarič, Gambardella[49] and Trompeta, Preiss,[50] which also reported the absence of MWCNT-induced mortality at a concentration of 0.1 g L^{-1}. However, they did observe bioaccumulation of black MWCNT aggregates in the intestinal tracts of *A. salina*, which were cleared within 24 hours, restoring normal functionality, indicating that there is no short-term impact on the organism's functionality.[50] For a concentration of 1 g L^{-1}, 100% mortality was observed, likely due to restricted movement in the higher-concentration environment[51] or, physical interference caused by the elongated shape of MWCNTs. The toxicity at this level is likely mechanical rather than chemical.[52] Nevertheless, such a high concentration is not environmentally relevant, and real-world exposure levels are expected to be far lower.

The exposure of *S. alba* to both MWCNT types resulted in less than 10% inhibition of germination across all concentrations when compared to the control. These findings are in line with the observations of Lin and Xing,[53] who reported no germination inhibition for radish, rape, ryegrass, lettuce, corn, or cucumber at MWCNT concentrations of up to 2 g L^{-1}. With regard to the impact on root growth, a stimulatory effect was observed at concentrations of 0.01 and 0.1 g L^{-1} for both materials (Table 2), with the oxidized form showing a stronger effect. However, at 1 g L^{-1}, an inhibitory effect was observed, with MWCNT causing twice the inhibition compared to MWCNT-ox. These results are consistent with those of Mondal, Basu,[54] who noted a beneficial effect on *Brassica juncea* (brown mustard) seeds at low MWCNT-ox concentrations compared to non-oxidized MWCNT. The stimulatory effect is likely due to MWCNT's ability to enhance water and nutrient absorption by plants.[55] However, at concentrations exceeding 0.1 g L^{-1}, these stimulatory effects diminish, leading to inhibition.[56]

Further, algal growth of *D. subspicatus* was inhibited following exposure to MWCNT and MWCNT-ox (Table 2). MWCNT-ox demonstrated lower inhibition rates in a concentration-dependent manner, with less than 10% inhibition at 0.01 g L^{-1} and 20% at 0.1 g L^{-1}. In contrast, the oxidized form demonstrated an inhibitory effect of approximately 25% at both concentrations. These differences in toxicity of the two forms of MWCNTs may be attributed to structural factors, whereby MWCNT-ox can interact more readily with biological molecules due to their reactive functional groups.[57]

In summary, at a concentration of 0.1 g L^{-1}, both MWCNT and MWCNT-ox exhibited similar toxicity levels, suggesting that general mechanisms like oxidative stress and interference with cellular functions—typical for MWCNTs—likely dominate.[58–60] Nevertheless, at higher concentrations, insufficient illumination or diminished accessibility of nutrients complexed with nanoparticle agglomerates may also contribute to the observed inhibition.[59] Identifying the exact source of inhibition can be challenging, as it often results from the complex interplay of multiple factors.

Table 2 Results of root growth inhibition (I_R) of *Sinapis alba* seeds, growth inhibition (I) of *Desmodesmus subspicatus*, and mortality of *Artemia salina* exposed to MWCNT and MWCNT-ox, respectively, for 72 h. Data are presented as means ± SD (n = 3). Means that do not share a letter within the same column are statistically significant at $p < 0.05$; different superscripted letters a, b, c indicate significant differences ($p < 0.05$) between samples within the same column

Sample	c (g L^{-1})	*A. salina* Mortality (%)	*S. alba* I_R (%)	*D. subspicatus* I (%)
MWCNT	0.01	0	$-4.7 \pm 3.4^{b,c}$	8.3 ± 3.4^{b}
	0.1	0	-16.2 ± 4.2^{c}	$20.3 \pm 3.2^{a,b}$
	1	100	22.0 ± 4.7^{a}	—
MWCNT-ox	0.01	0	-14.4 ± 10.4^{c}	25.2 ± 7.4^{a}
	0.1	13.3 ± 5.7	-14.7 ± 1.1^{c}	24.8 ± 4.1^{a}
	1	100	$10.2 \pm 8.4^{a,b}$	—

3.3 MOC samples preparation and characterization

As the tested MWCNT and MWCNT-ox were shown to be harmless to bacterial and eukaryotic growth but exhibited significant antibiofilm effect – an advantageous property for building materials – they were incorporated into MOC-based composites. Two types of MOC-based composites were prepared: a reference sample containing only MOC phase 5 and silica sand filler, and two modified samples doped with a small dosage of either MWCNT or MWCNT-ox.

The phase composition of the prepared samples was analyzed using XRD, with diffraction patterns shown in Fig. 4. In both samples, MOC phase 5 and silicon oxide in the form of quartz were detected. The presence of quartz originated from the utilization of silica sand as a filler. Even though the samples MOC-MWCNT and MOC-MWCNT-ox contained MWCNT and MWCNT-ox, these cannot be seen in the diffraction pattern, as their content is very low. The MOC phase 5 is considered crucial for the superior microstructural and mechanical properties of MOC-based composites.

SEM analysis of the fracture surfaces of the composites revealed a dense microstructure composed of interlocking, needle-shaped crystals typical of MOC phases (Fig. 5). These needle crystals are responsible for the excellent material performance of MOC-based materials. Silica sand filler grains are also visible in the micrographs. At higher magnifications, MWCNT and MWCNT-ox bundles were observed, formed due to their hydrophobic nature and propensity for agglomeration. In the future, it will be necessary to mitigate the agglomeration of the nanomaterials by using specific

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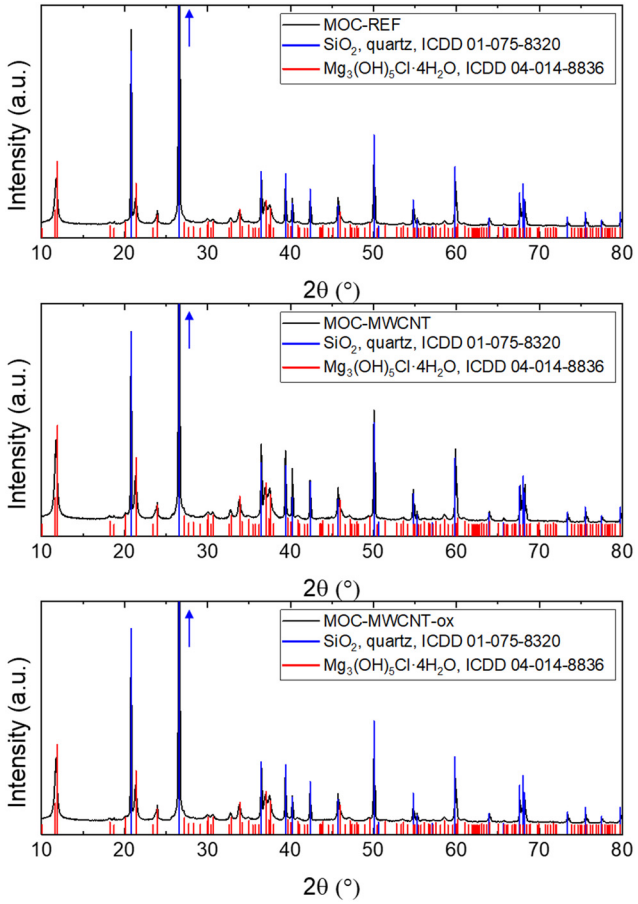

Fig. 4 Diffraction patterns of the samples MOC-REF (top), MOC-MWCNT (middle), and MOC-MWCNT-ox (bottom) obtained from XRD.

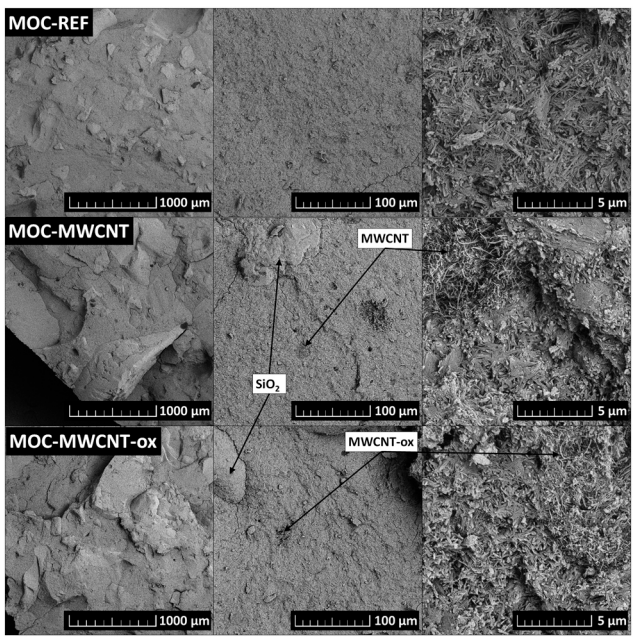

Fig. 5 SEM micrographs of the prepared MOC-based composites.

additives, such as surfactants,[61,62] or techniques, such as ultrasonication.[63]

During SEM analysis, EDS was employed to determine the chemical composition of the studied fracture surface. The detected elements included Mg, O, Cl, Si, and C (Fig. 6). Along with the qualitative analysis, the quantity of the individual elements on the fracture surface was studied using EDS. However, these results are influenced by the small area of the studied surface, and thus the elemental quantities are not presented as they do not reflect the true chemical composition of the samples.

3.4 Impact of MOC-MWCNT on prokaryotes and eukaryotes

After verifying the influence of MWCNT and MWCNT-ox on selected organisms, MWCNT-reinforced MOC samples were prepared and tested in terms of their ecotoxicological impact. First, we evaluated MOC sterility by testing the capture of microorganisms that can be cultivated by conventional cultivation methods. No microbial growth was observed after culturing the materials (MOC-REF as well as MOC-MWCNT and MOC-MWCNT-ox), in both solid and powder forms, in nutrient media, indicating that these materials are resistant to microbial contamination from the environment (Fig. S1†). This suggests that their surface and structure do not support microbial colonization, and that MOC is inherently sterile, without being influenced by the addition of MWCNT/MWCNT-ox. These results are promising for the use of MOC materials in applications where microbial resistance is critical, as microbial degradation is a major factor affecting material durability.[64]

Regarding bacterial interactions with the tested MOC materials, the results (Fig. 7) show that all three MOC samples inhibited bacterial growth in their surroundings. MOC-REF and MWCNT-reinforced MOC samples significantly reduced bacterial growth ($p < 0.001$). The addition of both types of MWCNTs to MOC enhanced the inhibition of *S. aureus* and *E. coli* growth ($p < 0.05$). In case of *P. aeruginosa*, only the addition of MWCNT significantly affected the growth, while the addition of MWCNT-ox to MOC had no observable effect.

Near-total inhibition of growth occurred for *S. aureus* and *E. coli*; *P. aeruginosa* showed lower growth inhibition compared to other tested bacteria, reaching a maximum of 30% growth compared to the control. This may be caused by a notable increase in medium pH caused by the MOC. The pH shifted from neutral to a basic environment (pH 9–10) during cultivation, consistent with other studies reporting MOC-induced alkalinity of pH 8–10.[65] Bacteria typically prefer neutral pH and can slightly alter the medium's pH through acidic or alkaline metabolites production during growth.[66] However, at pH levels of 9–10, bacterial growth may be affected due to disruptions in various cellular processes, including protein denaturation and membrane damage. That aligns with the observation that *P. aeruginosa* was the most resistant to the alkaline conditions.[67] An additional anomaly was observed in the behavior of *P. aeruginosa*—at pH 9 or higher in the presence of MOC, the bacterium did not

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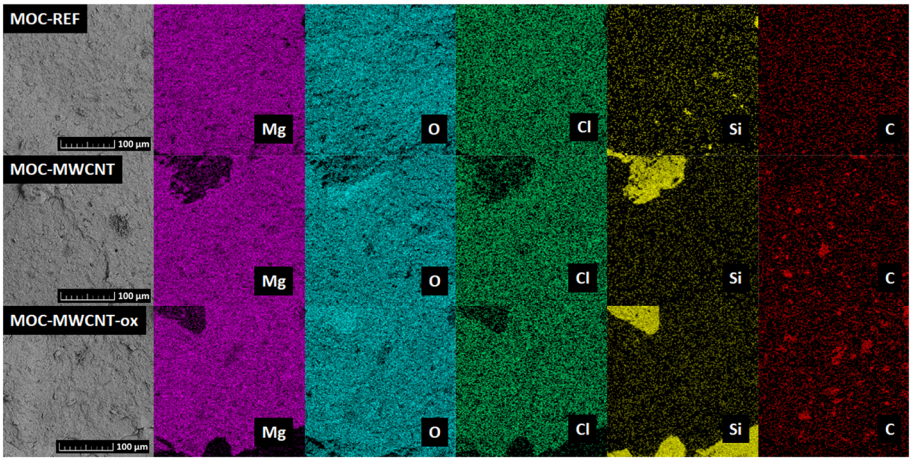

Fig. 6 EDS elemental maps of the prepared MOC-based composites.

produce its typical phycocyanin pigment, which gives the bacterial suspension a blue-green color (Fig. S2†). Phycocyanin is produced within an optimal pH range of 6.4–7.4 and is not produced at pH levels of 9 or higher.[68,69]

Further, we studied bacterial biofilm formation on the surface of MOC samples (Fig. 8). Both MOC-REF and MWCNT-reinforced MOCs inhibited biofilm formation in all tested bacteria, including the most durable one, *P. aeruginosa*. The biofilm formation in control samples reached values of 7.7 ± 0.0.2–8.5 ± 0.1 log (CFU cm^{-2}), while notably lower values were observed for MOC surfaces.

Biofilm formation on MOC-REF ranged between 1.8 ± 0.1 and 2.5 ± 0.0 log (CFU cm^{-2}), while MWCNT-/MWCNT-ox-reinforced MOC samples showed biofilm formation between 1.1 ± 0.2 and 2.3 ± 0.1 log (CFU cm^{-2}). The most significant effect was observed for MOC-MWCNT on *E. coli* ($p < 0.05$); no significant difference between the effect of MOC-REF and MOC-MWCNT-ox was confirmed ($p > 0.05$). SEM analysis revealed that only a minimal number of bacterial cells adhered to the surface of the tested samples (Fig. 9). The bacterial cells showed visible damage to their cell walls, which appeared wrinkled, and predominantly attached to areas with surface cracks or areas without nanotubes. This indicates that the surface of the material does not facilitate bacterial adhesion, preventing the formation of a continuous biofilm, a typical feature of the tested bacterial species.[70] The

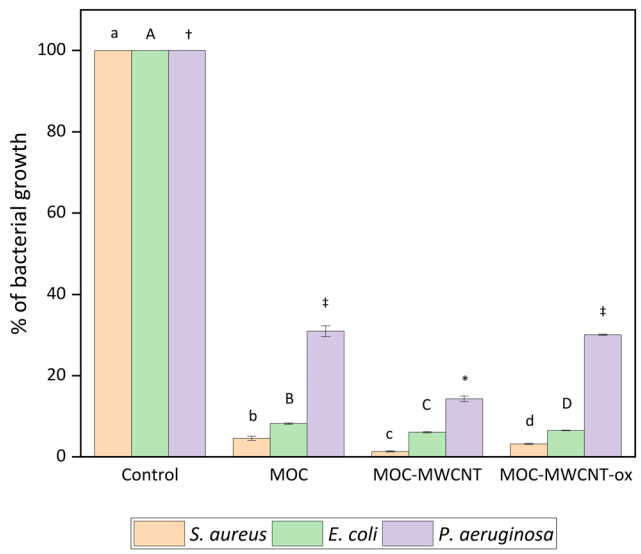

Fig. 7 Bacterial growth in the presence of MOC materials expressed as a percentage of bacterial growth compared to the control. Significant differences were represented with different letters or symbols (lowercase letters for *S. aureus*; uppercase letters for *E. coli*; symbols for *P. aeruginosa*). Means that do not share a letter or symbol within the same organism are statistically significant at $p < 0.05$ (ANOVA and Tukey's test with Bonferroni correction).

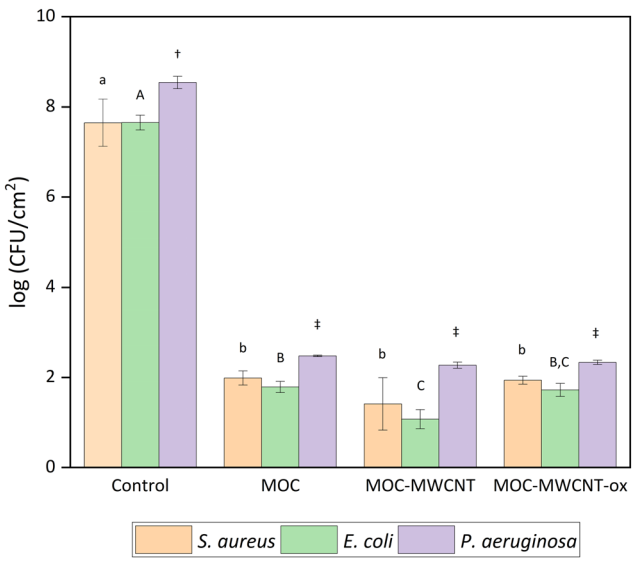

Fig. 8 Bacterial biofilm formation on MOC surface related to 1 cm^2. Significant differences were represented with different letters or symbols (lowercase letters for *S. aureus*; uppercase letters for *E. coli*; symbols for *P. aeruginosa*). Means that do not share a letter or symbol within the same organism are statistically significant at $p < 0.05$ (ANOVA and Tukey's test with Bonferroni correction).

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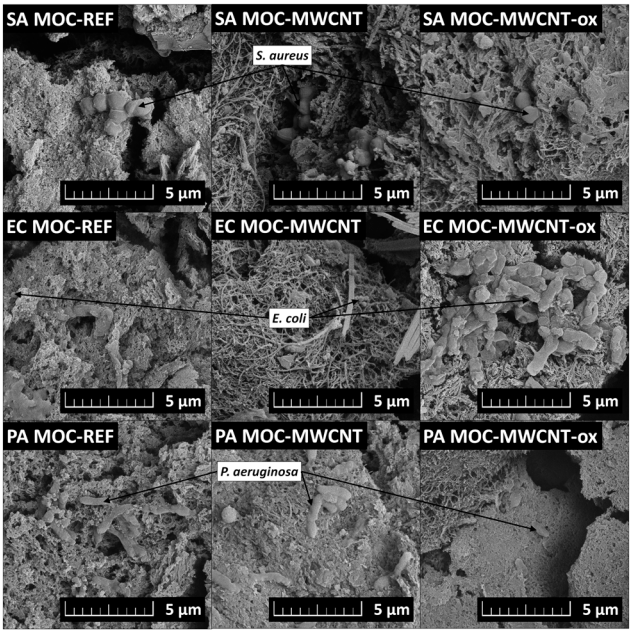

Fig. 9 SEM micrographs of the prepared MOC-based composites after exposure to prokaryotes *S. aureus* (SA), *E. coli* (EC), and *P. aeruginosa* (PA).

presence of prokaryotic organisms on selected samples was further confirmed by EDS. In the elemental maps (Fig. 10), areas containing *S. aureus* embedded in the MOC-MWCNT-ox composite showed increased carbon content.

To further investigate the mechanism of action of the tested materials on bacterial cells, oxidative stress induced by MWCNTs and MOCs was assessed using the model bacterium *S. aureus* (Table 3). H_2O_2 levels were measured as a marker of material-induced oxidative stress in bacteria, as H_2O_2 has the longest half-life compared to other ROS and reflect general changes in ROS levels over time. As the applied assay is based on luminescence detection, measurements of highly concentrated MWCNT and MWCNT-ox solutions are subject to greater statistical error and should be interpreted with caution. This bias arises from the blackness and increased turbidity of the solution, which absorb emitted light and thereby distort luminometric readings. Conversely, at lower concentrations, where these interfering effects are less pronounced, the assay revealed a notable increase in ROS production—by several tens of percent—in the presence of MWCNT and MWCNT-ox. However, for MOC composites (both reference and MWCNT-incorporated ones), the observed increases were not statistically significant.

Following the bacterial tests, the effect of MOC-MWCNT on *A. salina* mortality rate, *S. alba* germination and root growth inhibition, and *D. subspicatus* growth inhibition were investigated. We revealed that the toxicity of bulk MOC to *A. salina* was higher than that of the MOC-nanotube combination at a concentration of 1 g L^{-1} (see Fig. 11). One potential explanation is the increased compactness and stability of MOC in aqueous media when combined with nanotubes. At concentrations of 0.1 g L^{-1} and higher, a slight haze formed in the pure MOC solution, but not in the MWCNT mixture. An alternative explanation could relate to the chemical nature of MOC itself or residual substances from the preparation process.[71,72] At the lowest exposed concentration, all three materials exhibited identical toxicity (13%), indicating that the MOC is responsible for this effect.

To better understand the toxic effects on *A. salina*, it is important to consider not only mortality but also potential bioaccumulation, particularly in the intestinal tract. Despite the absence of bulk MWCNT in *A. salina* gut, our study revealed the presence of MOC-MWCNT and MOC-MWCNT-ox particles at the highest exposed concentration of 1 g L^{-1}

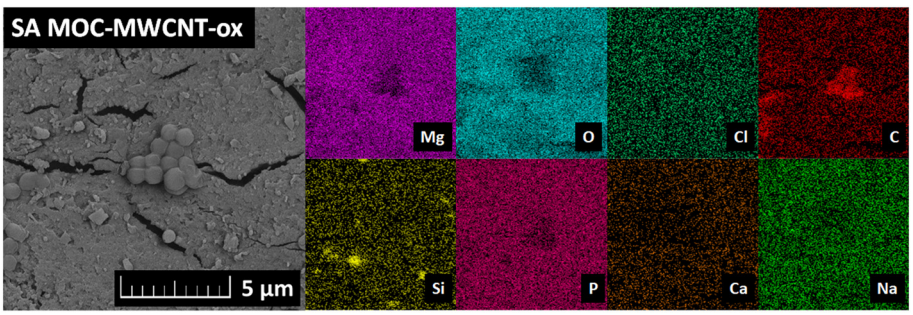

Fig. 10 EDS elemental maps of MOC-MWCNT-ox composite after exposure to *S. aureus* (SA).

Table 3 ROS production after cocultivation of *S. aureus* and tested materials: MWCNT, MWCNT-ox, MOC-REF, MOC-MWCNT, and MOC-MWCNT-ox

Sample	Control	MWCNT 1 g l^{-1}	MWCNT-ox 1 g l^{-1}	MWCNT 0.1 g l^{-1}	MWCNT-ox 0.1 g l^{-1}	MOC-ref	MOC-MWCNT	MOC-MWCNT-ox
ROS (H_2O_2) luminiscence (RLU)	18 964.0 ± 160.7	2057.3 ± 51.1	2125.3 ± 25.7	30 849.0 ± 161.6	32 444.7 ± 122.0	14 675.0 ± 160.1	15 389.3 ± 39.8	19 636.3 ± 21.2
Increase of ROS generation (average %)	0.0	−89.2	−88.8	62.7	71.1	−22.6	−18.8	3.5

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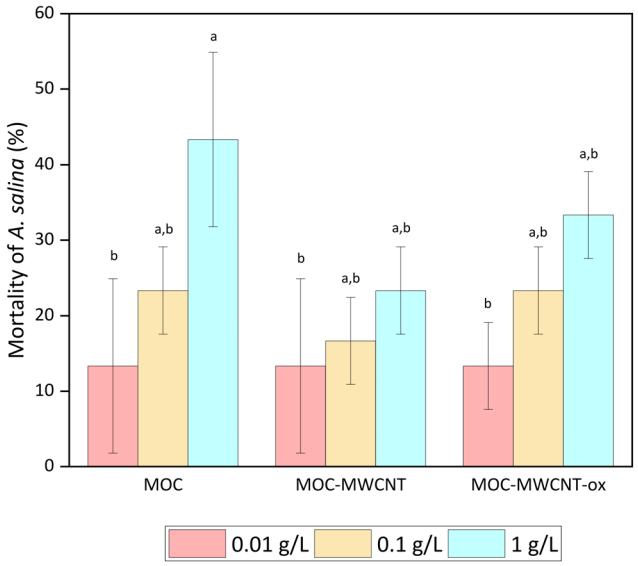

Fig. 11 *A. salina* mortality; means that do not share a letter are statistically significant at $p < 0.05$ (ANOVA and Tukey's test with Bonferroni correction).

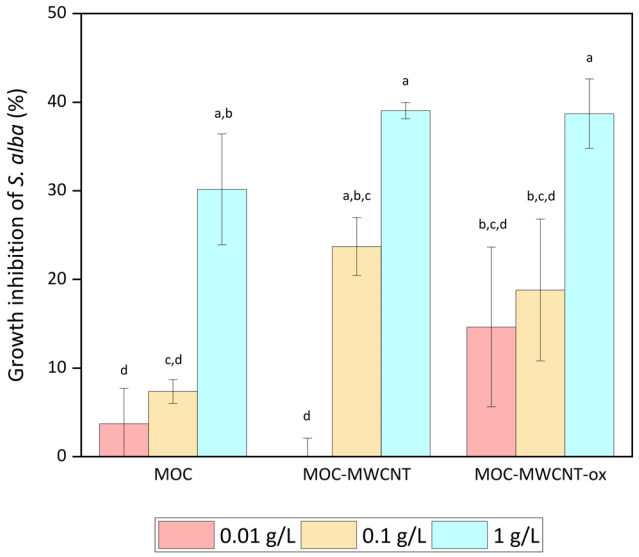

Fig. 13 *S. alba* root growth inhibition; means that do not share a letter are statistically significant at $p < 0.05$ (ANOVA and Tukey's test with Bonferroni correction).

(Fig. 12). The combination of MOC with nanotubes resulted in a shape modification that may have enhanced the particles' attractiveness to *Artemia*, thereby more closely mimicking natural food sources and increasing the probability of ingestion. Black particles were observed throughout the gut, indicating that they should be emptied over time. This is consistent with the findings of the study by Zhu, Luo[73] following exposure to oxidized MWCNT, where most of it was excreted after 72 h. Some particles were also attached to the body surface, which could cause mortality.[73]

Further, no significant inhibition of *S. alba* seed germination was observed, with inhibition levels not exceeding 10%. However, a notable inhibition of *S. alba* root growth was detected (Fig. 13), exceeding 30% at a concentration of 1 g L^{-1} for pure MOC and its combinations with MWCNT or MWCNT-ox. This effect could be attributed to the elevated magnesium ion levels in the solution. While magnesium is essential for plant growth and photosynthesis, excess magnesium can disrupt the mineral balance and adversely affect root development.[74,75] Inhibitory effects, approximately 20%, were also observed in MOC combinations with MWCNT and MWCNT-ox at a notably lower concentration of 0.1 g L^{-1}. At 0.01 g L^{-1}, significant root growth inhibition was only noted for MWCNT-ox. These results suggest that the combination of MOC and MWCNTs enhances the toxic potential in *S. alba* seeds at certain concentrations.

Next, we evaluated the inhibition of *D. subspicatus* growth by MOC samples. The potential for growth inhibition was assessed by direct cell counting of *D. subspicatus* following exposure to MOC and its combinations with MWCNT. While a concentration of 0.01 g L^{-1} MOC stimulated growth, a 10% increase in inhibition was observed at a concentration of 0.1 g L^{-1} compared to the control (Fig. 14). The combination of

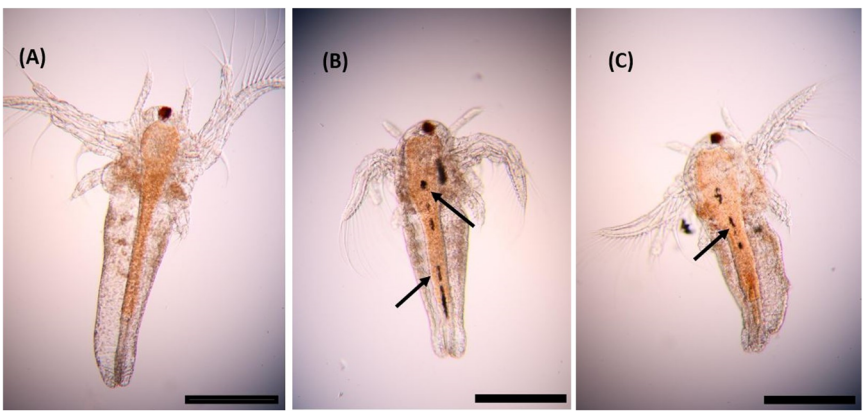

Fig. 12 Bioaccumulation of MWCNT by *A. salina* gut: (A) control, (B) accumulated MOC-MWCNT in 1 g L^{-1}, (C) accumulated MOC-MWCNT-ox in 1 g L^{-1}. Scale bars: 300 μm.

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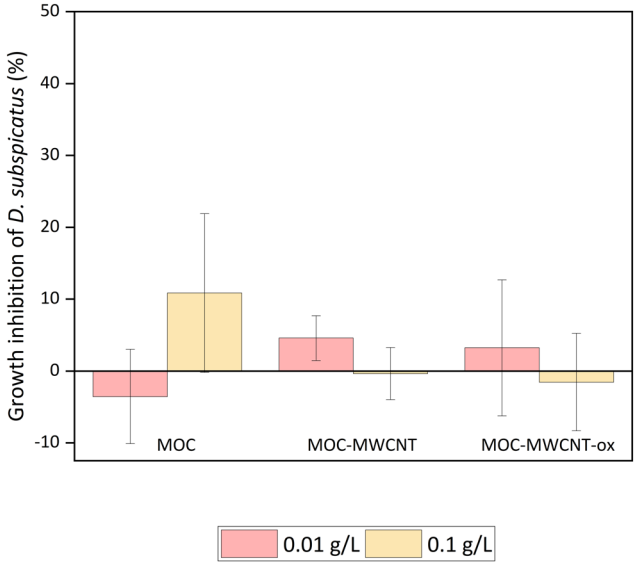

Fig. 14 *D. subspicatus* growth inhibition. Means are not statistically significant at $p < 0.05$ (ANOVA and Tukey's test).

MOC with nanotubes yielded unexpected antagonistic effects, with inhibition at both tested concentrations not exceeding 5%. The results were not found to be statistically significant. This suggests that the concentration of MWCNT/MWCNT-ox plays a key role in influencing algal growth inhibition.

At higher concentrations, the dispersion of pure MOC in BBM resulted in the formation of a slight haze, which may have created a shielding effect. This phenomenon is commonly observed when particles accumulate on the surface of organisms, leading to inhibition of photosynthetic activity.[76] However, the dispersion of MOC combined with MWCNT did not show this effect. Based on these findings, it can be inferred that the release of small quantities of these materials into the environment, such as through degradation,[77,78] is unlikely to significantly impact freshwater algae like *D. subspicatus*.

3.5 Summary of results

To summarize all the results, the following conclusions were made. MWCNT and MWCNT-ox, at concentrations of 1 g L^{-1}, 0.1 g L^{-1}, and 0.01 g L^{-1}, did not significantly influence bacterial growth, although they provided strong antibiofilm effects. No significant difference was observed between the non-oxidized and oxidized forms of MWCNT in their effects on the tested bacterial strains. Regarding the tested eukaryotes, both MWCNT and MWCNT-ox slightly influenced growth and viability. For *A. salina*, no significant mortality or bioaccumulation was detected at 0.01 and 0.1 g L^{-1}, but the highest concentration of 1 g L^{-1} caused total mortality. *S. alba* root growth inhibition varied by concentration, with a stimulatory effect observed at 0.01 and 0.1 g L^{-1} for both MWCNT and MWCNT-ox, while the 1 g L^{-1} concentration resulted in significant inhibition, with MWCNT showing twice the inhibitory effect compared to MWCNT-ox. Low inhibition levels were observed for *D. subspicatus* growth, with a concentration-dependent effect.

Additionally, the prepared MOC samples inhibited bacterial growth in the surroundings and provided strong antibiofilm properties, primarily due to the MOC itself. No significant difference between MOC-MWCNT and MOC-MWCNT-ox effects was detected. For the tested eukaryotes, MOC samples affected growth and viability, with bulk MOC showing higher toxicity to *A. salina* than its combination with nanotubes. For *S. alba*, root growth inhibition exceeded 30% at a concentration of 1 g L^{-1}, likely due to elevated magnesium levels. In the case of *D. subspicatus*, no significant toxic effects were observed for MOC or its combinations with nanotubes.

Based on these results, we consider the prepared MOC materials to be environmentally safe, with minimal ecological impact. As MWCNT characteristics may vary between organisms, we recommend testing the toxicity of both MWCNT additives and MOC-reinforced composites in specific ecotoxicological assessments before their application.

Conclusion

MOC/MWCNT composites present an eco-friendly alternative to PC-based composites. The proposed composite system is easily synthesizable, manifests high values of mechanical parameters, and, due to the utilization of the functional additive, MWCNTs, it provides sufficient water resistance, which is a crucial application potential-limiting factor in the case of MOC-based binders. Given the intended use in construction, it is critical to study the ecotoxicological impacts of these composites. To address this need, the effects of MOC-MWCNT and MOC-MWCNT-ox on six selected organisms were investigated. The acquired findings confirmed that the characteristics and concentrations of MWCNTs play a crucial role in their interactions with both prokaryotes and eukaryotes. Overall, the addition of MWCNTs to MOC did not dramatically increase inhibitory effects on the tested organisms, though specific differences were observed depending on the organism and MWCNT concentration. Notably, the tested MOC samples displayed strong antibiofilm properties, suggesting low risk of microbial degradation. These results highlight the potential of MWCNT-reinforced MOC as environmentally safe alternatives to traditional building materials. However, it is recommended to verify the toxicity of specific CNTs and their reinforced composites prior to application, as the effects may vary depending on the physicochemical properties of the CNTs, their concentrations, and the characteristics of the target organisms. After this, the upscaling of the MOC-MWCNT and MOC-MWCNT-ox production, and their use in construction applications such as flooring or the fabrication of construction elements, such as panels or specific prefabricated elements, will be possible. This solution will provide a competitive, eco-friendly material able to replace conventional construction composites with increased microbial resistance.

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Data availability

According to open science principles, data can be found at DOI: **https://doi.org/10.5281/zenodo.13935318**.

Author contributions

Simona Lencova: conceptualization, methodology, investigation, data curation, formal analysis, writing – original draft, writing – review & editing. Jana Kofronova: methodology, investigation, visualization, data curation, formal analysis, writing – original draft. Vaclav Peroutka: methodology, investigation, formal analysis, visualization, writing – original draft, writing – review & editing. Anna-Marie Lauermannova: writing – original draft, investigation, data curation, formal analysis, visualization, writing – review & editing. Adela Jirickova: writing – original draft, investigation, data curation, formal analysis, writing – review & editing. Michal Lojka: investigation, data curation. Ondrej Jankovsky: project administration, writing – original draft, resources, supervision. Radek Vurm: investigation, data curation.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was financially supported by the Czech Science Foundation (project 23-05194M), while the infrastructure has been utilized in the frame of project No. CZ.02.01.01/00/22_008/0004631. Materials and technologies for sustainable development within the Jan Amos Komensky Operational Program financed by the European Union and from the state budget of the Czech Republic.

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