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Graphene enhanced detoxification of wastewater rich 4-nitrophenol in multistage anaerobic reactor followed by baffled high-rate algal pond

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ABSTRACT

The presence of 4-nitrophenol (4-NP) in the wastewater industry causes toxicity and inhibition of the anaerobic degrading bacteria. The anaerobes in the multistage anaerobic reactor were loaded by 30.0 mg/gVS Graphene nanoparticles (MAR-G_n) as an electron acceptor to detoxify wastewater industry. The half maximal inhibitory concentration (IC_{50}) was reduced from 455 ± 22.5 to 135 ± 12.7 μg Gallic acid equivalent/mL at 4-NP loading rate of 47.9 g/m^3 d. Furthermore, 4-NP was decreased by a value of 83.7 ± 4.9% in MAR-G_n compared to 65.6 ± 4.8% in control MAR. The 4-aminophenol (4-AP) recovery was accounted for 44.8% in the MAR-G_n at an average oxidation-reduction potential (ORP) of − 167.3 ± 21.2 mV. The remaining portions of 4-NP and 4-AP in the MAR-G_n effluent were efficiently removed by baffled high rate algal pond (BHRAP), resulting in overall removal efficiency of 91.6 ± 6.3 and 92.3 ± 4.6%, respectively. The *Methanosaeta* (52.9%) and *Methanosphaerula* (10.9%) were dominant species in MAR-G_n for reduction of 4-NP into 4-AP. Moreover, Chlorophyta cells (*Chlorella vulgaris, Scenedesmus obliquus, Scenedesmus quadricauda and Ulothrix subtilissima* were abundant in the BHRAP for complete degradation of 4-NP and 4-AP.

1. Introduction

The majority of chemical industries produce wastewater-rich xeno-biotech compounds that are recalcitrant and hardly biodegraded in biological systems (Ismail et al., 2019a, 2019b). 4-nitopheniol (4-NP) is an aromatic compound found in the wastewater generated from insecticides, pesticides, dyes, drugs, and explosives industries (Schackmann and Müller, 1991). This compound causes toxicity and severe environmental pollution problems where it accumulates in the sediment of the water streams and negatively affects the living fauna (Kulkarni and Chaudhari, 2007; Ismail et al., 2021). Fortunately, carbon materials are employed to remove 4-NP from wastewater due to the easier manipulation of their surface's physical and chemical properties (Gar Alalm et al., 2016a, 2016b). Moreover, the carbon materials enjoy functional groups that can be used as a catalyst for the detoxification of hazardous pollutants (Amezquita-Garcia et al., 2015; Alalm et al., 2015). However, the efficiency of activated carbon fibers achieved removal efficiency of only 17–30% for 4-NP due to clogging of pores by adsorbents. However, hybrid biological technology based on activated carbon remains a sustainable and attractive solution for removing 4-NP from wastewater, as reported earlier by Amezquita-Garcia et al. (2016).

Activated carbon has Quinone groups in its structure (Pereira et al., 2010) facilitated their use as redox mediator during the biological detoxification process resulting in an efficient reductive transformation

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process of hazardous pollutants. Redox-active functionalities of activated carbon improved the reduction efficiency of 4-NP in anaerobic reactor treating wastewater industry (Amezquita-Garcia et al., 2016). Clogging the pores is the main serious problem for the application of activated carbon due to excessive microbial growth, which highly reduces the efficiency of the materials (Amezquita-Garcia et al., 2013). Therefore, Graphene nanoparticles (G_n) would overcome such problem and enhance the reduction efficiency of 4-NP (Nasr et al., 2021). The G_n will exhibit more effectively electron transfer via Quinone groups and/or delocalized electrons to the nitro- group, transforming nitro- to their amino group.

Immobilization of anaerobes degrading 4-NP on G_n is a novel approach due to an increase of surface area and electron transfer (Elreedy et al., 2019). The dual interaction of the G_n and anaerobes would undoubtedly enhance the reduction efficiency of the 4-NP onto 4-AP. Graphene oxide was reported to be an electron shuttle increasing the redox conversion of the azo-dyes and the nitro-aromatic compounds (Colunga et al., 2015). The nanoparticles and nano-composite materials were efficiently used for reduction of 4-NP into 4-AP (Shah et al., 2021; Sun et al., 2019). Silver nanoparticles catalyzed the reduction of 4-NP into 4-AP with sodium boro-hydride (Kästner and Thünemann, 2016). Pd, Pt, Ag, and the bi-metals PdPt in nano-scale was employed from removal of 4-NP from wastewater industry (Capeness et al., 2019). Palladium (Pd) nanoparticles were efficient for the reduction of 4-AP (Lee et al., 2021).

The anaerobic treatment is mainly employed as a reductive process for converting 4-NP onto 4-AP in the treated effluent, which should be removed to be applicable for safe discharge into the environment. The phyco-remediation process is an acceptable technology for removing the 4-AP from anaerobically pretreated effluent (Xu et al., 2015). Algal mixed culture fauna and strains had the capability of metabolizing phenolic compounds existing in the wastewater (Lika and Papadakis, 2009). Klekner et al. Klekner and Kosaric (1992) found that *Chlorella sp*. completely biodegraded 1000 mg/L of 2,4-dimethyl phenol using a cultivated algal cells concentration of 4.0 g/L. *C. fusca* efficiently removed biphenyl, l,2,4-dinitrophenyl, o, m, and p-nitro phenol from wastewater (Hirooka et al., 2003a). The algal species transformed the phenol into pyruvate and CO_2 (Semple and Cain, 1996). *C. fusca* completely biodegraded 80 μM of bisphenol (Hirooka et al., 2005). 50% of phenols were removed by *C. pyrenoidosa* (Malvis et al., 2019). *Leptolyngbya* sp., reduced the phenol concentration of 100 mg/L by a value of 98% (Guha Thakurta et al., 2018). However, Xu et al. (2015) found that the degradation of 4-NP by algae (*Chlorella pyrenoidosa*) was not significant. This was not for Hirooka et al. (2006), who completely removed 2,4-dinitrophenol (2,4-DNP) from wastewater industry by cyanobacteria (*A. variabilis and A. cylindrical)*. 2,4-DNP, O- and p-nitrophenol was removed by *Anabaena variabilis* (Hirooka et al., 2003a). The less toxic by-products of 4-AP was removed from the treated effluent by Hirooka et al. (2006), who found that the 2-AP was eliminated by *C. reinhardtii, A. cylindrical,* and *Cyanobacterium*.

An integrated system consists of a multistage anaerobic reactor loaded with mixed culture with G_n (MAR-G_n) and baffled high rate algal pond (BHRAP) was designed and fabricated at pilot scale for continuous treatment of wastewater industry rich 4-NP. Such an integrated system was not previously reported in the literature. The existence of nitro-group in the 4-NP is highly electron withdrawing properties which is readily transformed to their corresponding less toxic amines (4-AP) in the MAR-G_n module. The subsequent BHRAP will efficiently transform the 4-AP onto ammonia, carbon dioxide, and water. The algal cell unit was divided by vertical baffles to increase oxygen proliferation, enhance the growth of aerobic bacteria and avoiding the presence of dead zones. The designed baffles will increase the interaction between the substrate and microbial/algal diversity, and raise the flow turbulence to overcome the formation of anaerobic zones in the algal unit. Baffling the algal unit will reduce the mass transfer limitations, shear stress conditions and increase the flocculation of algal cells to facilitate light penetration (Osama et al., 2020). Baffled duck weed pond system achieved a superior results for removal of 1,4 dioxane (69.3%) compared to classical one (20.8%) at an HRT of 4.0 days (Osama et al., 2020). This was linked to high micr-organisms' diversity and growing of various types of aerobic bacteria in the duck weed roots and basin (Osama et al., 2021). Furthermore, baffling of the module improved the oxygenation of the bulk liquid up to 5.4 ± 2.5 mgO_2/L compared to 1.3 ± 0.02 mg O_2/L in classical duck weed unit (Bassuney and Tawfik, 2017; Allam et al., 2016b).

Most of the earlier studies for treatment of wastewater rich 4-NP was mainly carried out using pure culture bacteria and carbon media as a redox materials. It is hardly, to find in literature a continuous system containing G_n for treatment of wastewater rich 4-NP. Immobilization of G_n on the mixed culture anaerobes treating wastewater industry containing 4-NP in a continuous operation mode is the novelty of this research. Moreover, identification of consortia responsible for reduction of 4-NP in the presence of G_n is quite new. Obtaining natural and new algal cells capable for removing of the 4-NP and 4-AP is beneficial from scientific point of view for real application of high rate algal pond systems for removal of hazardous compounds from wastewater industry.

This study aims to (1) assess the efficiency of the integrated/hybrid technology for the treatment of wastewater containing 4-nitophenol, (2) investigate the role of G_n as a redox mediator for reduction of 4-nitrophenol (3) study the effect of NH_4-N/P ratio on the degradation of 4-NP and, (4) identify the algal and microbial community degrading 4-NP and 4-AP showing the role of G_n for the acceleration of the abiotic reductive activity.

2. Materials and methods

2.1. Wastewater composition

Industrial wastewater was collected daily from the main hole of the end-off pipe effluents of chemical industries in New Burg El-Arab city, Alexandria, Egypt. Dyes, insecticides, pesticides, and drugs industries are the major facilities in this area where most pollutants are xenobiotic compounds, particularly 4-nitrophenol (4-NP). The pH value of the influent wastewater was varied from 7.1 to 8.5, with an average value of 7.9 ± 0.27. The COD was highly fluctuated from 2623to 9950 mg/L due to the changing of the facility products and utilizing raw materials. The wastewater contains a nutrient for microbial growth where the ammonia (NH_4-N) and phosphorus (P) were in an average value of 186.2 ± 52.9 mg/L and 7.3 ± 1.5 mg/L. The average NH_4/P ratio was 27.2 ± 10.5 in the influent wastewater. The IC_{50} of the wastewater was 455 ± 22.5 μg Gallic acid equivalent/mL.

2.2. Multistage anaerobic reactor (MAR) and baffled high rate algal pond (BHRAP)

A pilot-scale of the multistage anaerobic reactor (MAR) with a volumetric capacity of 27.6 L was installed at the wastewater collection area and continuously supplied with wastewater (80 L/d) (Fig. 1) by Peristaltic Pumps (Heidolph model-Germany). The module was fabricated and manufactured from transparent pre-spex and supplied with 15.0 L mixed culture harvested from a full-scale anaerobic digester in Al-Gabl Al-Asfer, Giza, Egypt. The inoculum sludge represents 54.4% of the total reactor volume with VS/TS ratio of 0.63. The total and volatile solids content was 34 and 21.4 g/L, respectively. The total sludge loading rate amounted to 321.0 g/reactor volume.

The MAR module is a rectangle with dimensions of length (78.5 cm), width (13 cm), and depth (27 cm). The unit comprises four compartments 1st, 2nd, 3^{rd,} and 4^{th,} and ended with a settling tank for separating the solids from treated wastewater. Each compartment is divided into two chambers, the 1st small with a width of 3.0 cm and height of 27 cm to receive the incoming wastewater. The baffle is bent ($\theta = 60^o$) to allow the substrate to contact the anaerobes situated in the 2nd chamber

Fig. 1. Experimental set-up of the integrated system treating wastewater containing 4-nitrophenol.

with 8 cm width. The volume of each compartment is 5.52 L. The module is supplied with an opening on the top to receive and collect the gases. Moreover, the unit is equipped with a side opening to collect samples for profile analysis and excess sludge discharge. The setter is located at the end of the reactor with a capacity of 5.52 L with an opening for discharge of the excess sludge.

The MAR was continuously operated for 300 days at a local ambient temperature (17–33 ºC) and fed with a flow rate of 80.0 L/d resulting in a hydraulic retention time (HRT) of 8.3 h. The mixed culture bacteria were immobilized on the prepared Graphene nanoparticles (G_n) at a dose of (30.0 mg/gVS). The intentional discharge of the excess sludge from MAR modules was periodically carried out avoiding washout of the biomass. This operational mode maintained the sludge concentration in the reactors at a level of 21.4–25 gVS/L and sludge residence time (SRT) of 83 days.

The baffled high rate algal pond (BHRAP) was manufactured from transparent Perspex to allow the penetration of the natural light to the algal cells for growth and enhance the photosynthesis process. The unit was divided into three equal compartments with a total volume of 153 L. Length, width, and depth of the unit was 1.7 × 0.3 × 0.3 m, respectively. The unit was initially operated without the inoculum of culture algal cells which was formed naturally. The BHRAP system received the anaerobic effluent. The unit was operated at HRT of 1.9 d., temperature of 17–33 ºC and daily 16/8 h light/darkness as shown in Fig. 1. HRAP are normally operated at HRT (2–6 d) (Muñoz and Guieysse, 2006; Allam et al., 2016a).

2.3. Graphene nanoparticles preparation and characterization

Graphite powder was oxidized by Hummer's method for the preparation of Graphene oxide (Tawfik et al., 2021). 30 g Graphite powder was mixed and carefully stirred in an ice bath with 700 mL H_2SO_4 conc. 90 g of $KMnO_4$ was slowly added at temperature < 20 °C. The water bath temperature was increased up to 40 °C, and the mixture was vigorously stirred for time of 0.5 h. 1.5 L H_2O was added during stirring for 15 min at 95 °C. 500 mL of water and 150 mL H_2O_2 (30%) was added to the reaction medium. The color changed directly from dark brown to yellow. The pH of the mixture was neutral after washing several times with deionized water. The solution was carefully filtered and further centrifuged with 1:10 HCl to remove metal ions, and the graphite oxide was dried in an oven at temperature of 60 °C. The prepared graphite oxide was dispersed in dist·H_2O (200 mg/100 mL) in an ultrasonic bath for 2.0 h. to obtain exfoliated Graphene oxide. NaOH was used to adjust the pH value at 10. Hydrazine monohydrate (20 mL)

was carefully added at 95 ºC and continuously stirred for 24 h. The reduced Graphene oxide was filtered as a black powder and washed with dist·H_2O to remove the excess hydrazine. The harvested Graphene nanoparticles (G_n) were dried in a vacuum oven at a temperature of 60 ºC. Scanning Electron Microscope (SEM), energy-dispersive X-ray spectroscopy (EDX), and Transmission electron microscopy (TEM) for Graphene are presented in Fig. 2**.** The elemental analysis of G_n by EDX consisted of carbon (51.51 wt%) and oxygen (48.49 wt%). The presence of oxygen atoms in the G_n structure indicated that the reduction process of Graphene oxide to Graphene was not yet perfect because there are other oxygen groups in the Graphene structure. The atom ratio of C: O in G_n was 1.06.

2.4. Analytical methods

The efficiency of MAR and BHRAP modules was assessed by analyzing the influent wastewater and their treated effluents. The pH value was measured by JENWAY 3510 device. The CODt was measured by HACH method. NH_4–N, and TP was measured based on the methods described by APHA (2005). Volatile fatty acids (VFAs), 4-nitrophenol, and 4-aminophenol were determined by HPLC (Agilent 1260). Half maximal inhibitory concentration (IC_{50}) was determined by the method described by Velioglu et al. (1998) and was estimated as µg gallic acid equivalent (GAE). The extracellular polymeric substances (EPS) were determined based on the method described by Ismail et al. (2019b). The morphology of the G_n and sludge samples was determined using high-resolution SEM and EDX (JEOL JSM-6010LV, Japan). Fourier Transform infrared spectrophotometer (Spectrum 65 FT-IR, Perkin Elmer, USA) was used analysis functional groups.

2.5. Structure of microbial community

Identification of anaerobes immobilized on Gn was done as described by Ismail et al., (2021). DNA extraction was carried out based on the method described earlier by Fawley et al. Fawley and Fawley (2004). PCR amplification and 16S rRNA sequencing was conducted for the extracted DNA (S1). OTU assignments and statistical analysis and sequences were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) software (Wu et al., 2010). Taxonomy visualization was obtained by microbiome analyst (Dhariwal et al., 2017).

3. Results and discussion

3.1. Effect of addition graphene nano-particles on the 4-nitrophenol degradation efficiency in the multistage anaerobic reactor

The COD influent was highly varied from 2623 to 9950 mg/L in the wastewater industry. The anaerobes utilize those organics as electron donors to reduce 4-nitrophenol (4-NP) into 4-amino-phenol (4-AP). However, the reduction efficiency of 4-NP could be sustained and improved in the presence of Graphene nano-particles (G_n). The reduction efficiency of 4-NP in the classical multistage anaerobic reactor (MAR) versus multistage anaerobic reactor supplemented with Graphene nanoparticles (MAR-G_n) are shown in Fig. 3a.

Immobilization of anaerobes on G_n in the MAR-G_n improved the reduction efficacy of 4-NP by a value of 18%, which equivalent to 2.6 mg4-NP/L in the treated effluent. Lower residual values of 2.8 ± 0.018 mg4-NP/L were achieved in the treated effluent of MAR-G_n at OLR of 14.1 gCOD/m^3 d. The OLR of 19.4 gCOD/m^3 d imposed to the classical MAR resulted a higher residual values of 5.4 ± 0.027 mg4-NP/L in the effluent. Graphene nanoparticles (G_n) facilitates the electron transfer from the degradable organics onto 4-NP to be reduced into 4-AP by the existing anaerobes (Ağdağ and Sponza, 2005). The G_n enjoys redox mediator functional groups as well, which enhance the transfer of interfacial electrons. Colunga et al. (2015) found that immobilization of anaerobes on 5.0 mg/L Graphene oxide enhanced the reduction of nitro aromatic compounds in the presence of sulfide as electron donor. Similarly, Graphene oxide improved the azo-dye degradation efficiency

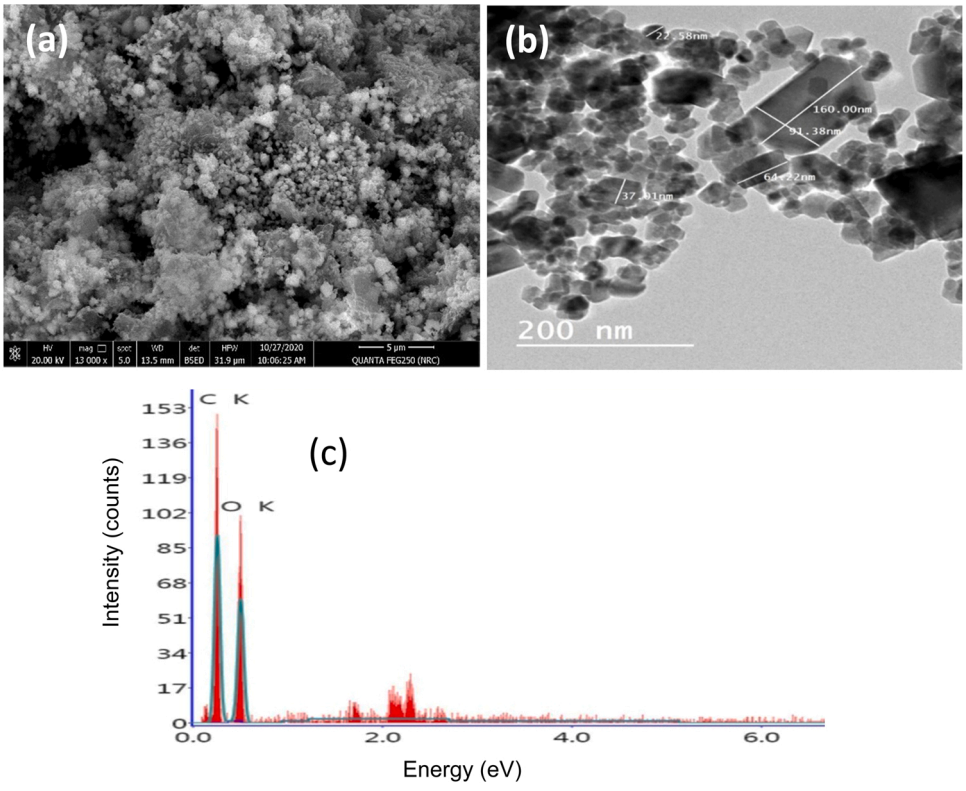

Fig. 2. Scanning electron microscope (SEM) (a), transmission electron microscopy (TEM) (b) and energy-dispersive X-ray spectroscopy (EDX) (c) of Graphene nanoparticles (G_n).

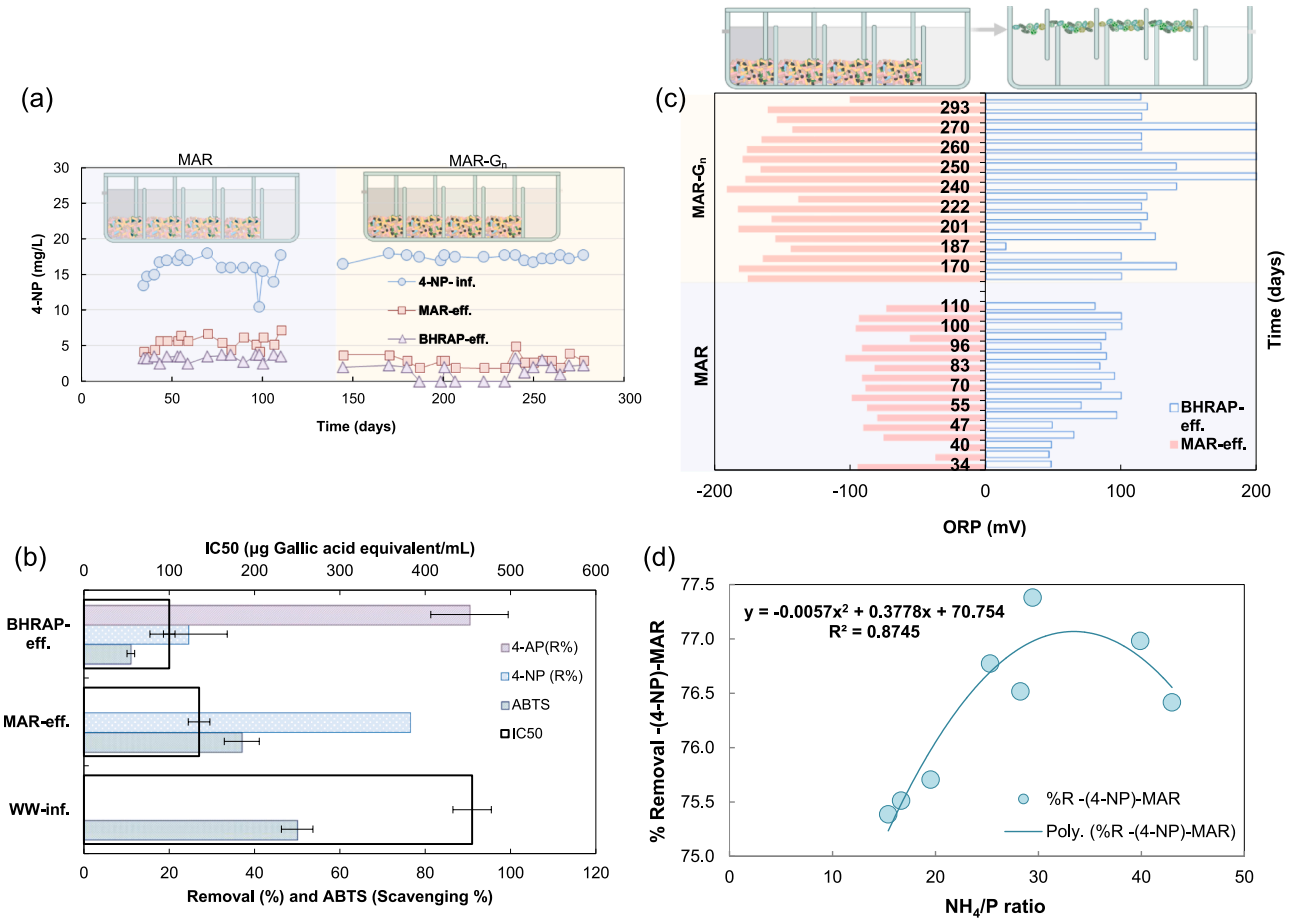

Fig. 3. (a) Classical multistage anaerobic reactor (MAR) versus multistage anaerobic reactor supplied with graphene nanoparticles (MAR-G_n) for removal of 4-nitophenol from wastewater industry. (b) Half-maximal inhibitory concentration (IC_{50}), antioxidant activity-ABTS (scavenging %), 4-NP removal and 4-AP recovery (% R) in the integrated system. (c) Oxidation-reduction potential (ORP) of the treated effluents of MAR/BHARP versus MAR-G_n/BHRAP. (d) Effect of NH_4/P ratio on the removal of 4-nitrophenol.

by 2–3.6 folds due to an increase of electron shuttle and subsequently the redox conversion efficiency. Activated carbon-containing redox--active functionalities enhanced as well the reduction of 4-NP in the anaerobic module (Amezquita-Garcia et al., 2016). The majority of 4-NP was anaerobically biodegraded (83.7 ± 4.9%) in the MAR-G_n module resulting in an effluent quality of 2.8 ± 0.08 mg/L at NP-loading rate of 47.9 g/m^3 d. However, the 4-AP recovery was accounted for 44.8 ± 1.04% in the treated effluent of the MAR-G_n. This indicates that adsorption onto sludge containing G_n could play a role for removal of 4-NP. This phenomenon will be considered in future research. Higher removal efficiency (94%) of nitrophenols (2-NP and 4-NP) was recorded at loading rates of 910 and 790 mg/L.d. in UASB reactor (Donlon et al., 1996). 4-nitrophenol removal rate of 0.36 mg/L.d., with 82% removal efficiency, was reported by Haghighi Podeh et al. (1995) in an anaerobic reactor. Anaerobes in a fixed-film digester adapted for removal of 2-NP achieved a maximum reduction efficiency of 30 mgNP/L.d., (Giot et al., 1995). Nitrophenol removal was > 90% in an anaerobic fluidized-bed unit fed with synthetic wastewater at loading rate of 900 mg mono-nitrophenols/L. d., (Tseng and Lin, 1994). The MAR-G_n module produced a biogas productivity of 2.04 NL/d., with CH_4 content of 55% which was higher than those recorded for classical MAR (1.48 NL/d., biogas with 50% CH_4 content). Delia Teresa Sponza et al., (Sponza, 2005) obtained comparable data for biogas (2.48 L) and CH_4 of 57% at p-Nitrophenol loading rate of 5.78 mg/L.d., which slightly dropped to 43% at a loading rate of 9.63 mg/L.d. The presence of 4-NP in the wastewater causes a toxicity and inhibition effect of the anaerobes degrading bacteria. However, the immobilization of anaerobes on G_n and existing readily biodegradable organics in the wastewater facilitate the biodegradation of the 4-NP and reduce the toxicity effect. The half maximal inhibitory concentration (IC_{50}) was significantly reduced from 455 ± 22.5–135 ± 12.7 µg Gallic acid equivalent/mL in the treated effluent of MAR-G_n due to the reduction of 4-NP onto 4-AP as shown in Fig. 3b. ABTS was increased by 13%. Methanogenic activity was reported to be inhibited at IC_{50} of 26.5 mg *p*-NP/L (Kuşçu and Sponza, 2005).

The 4-NP removal rate amounted to 42.1 g/m^3.d., at a loading rate of 47.9 g/m^3.d., in MAR-G_n and dropped to 29.9 g/m^3.d in classical MAR. Furthermore, the 4-AP recovery rate of 16.1 g/m^3.d was obtained in MAR-G_n and reduced to 9.5 g/m^3.d in the classical MAR. The removal efficiency of p-nitrophenol using sequential anaerobic migrating blanket reactor was reduced from 94% to 85% at increasing p-nitrophenol loading rates from 0.96 to 9.63 g/m^3.d (Sponza, 2005). Donlon et al. (1996) reported that 22% of the produced less toxic 4-AP was biodegraded in the anaerobic reactor fed with wastewater-rich nitrophenols, and the conversion of aminophenol into methane is occurred using homoacetogenic fermentation process. The activities of methanogenesis were promoted where the pH value of the reaction medium increased from 7.8 ± 0.49 (influent) to 8.3 ± 0.27 (anaerobic effluent) due to utilization of volatile fatty acids (VFAs) as an electron donor for anaerobes to reduce the 4-NP into 4-AP. The VFAs (acetate, propionate, butyrate, and valerate) was removed by values of 56 ± 4.5, 42 ± 3.2, 48 ± 3.8, and 32 ± 2.3%, respectively in the MAR-G_n. VFAs promoted the release of metabolites necessary for the reduction of the nitro-aromatic compounds (Allam et al., 2016b). 80% of the p-nitrophenol

was converted into p-aminophenol in anaerobic sequential batch reactor in the presence of propionic acid, which increased the ORP of the reaction medium up to − 350 mV (Buitron et al., 2003). Likely, most of 4-NP was anaerobically reduced into 4-amino phenol (4-AP) in the UASB reactor inoculated with anaerobic granular sludge immobilized on the activated carbon cloths by HNO_3 and functionalized with anthraquinone-2,6-disulfonate at HRT of 8.0 h., and loading rates of 2.8 gCOD/L.d., (García-Rodríguez et al., 2019). In another study by Buitrón et al. (2005) P-nitrophenol was completely removed (100%) and partially converted into 75% of P- aminophenol in An-SBR at all PNP:PA ratios except 1:5. The presence of G_n in the sludge of the MAR-G_n reduced the oxidation-reduction potential (ORP) in the treated effluent, as shown in Fig. 3c. The ORP was averaged at a level of − 167.3 ± 21.2 mV for MAR-G_n and − 80.829 ± 22.736 mV for classical MAR. This was the reason for improving the reduction efficiency of 4-NP into 4-AP due to decrease of the redox potential of the reaction medium in the presence of G_n. p-nitro-phenol was removed by value of 95% in the anaerobic migrating blanket reactor at ORP of − 360 mV (Ağdağ and Sponza, 2005).

The required N and P for acetate conversion into methane is 3–6 kg N/ton and 0.5 kg/ton depending on the imposed SRT (Hussain et al., 2015). The results in Fig. 3d show the biodegradation efficiency of 4-NP in MAR-G_n at different NH_4/TP ratios. The removal efficiency of 4-NP was slightly increased from 75.3% to 77.4% at increasing the NH_4/P ratio from 15.4 to 29.4, respectively. Nevertheless, the 4-NP removal efficiency was dropped at increasing the NH_4/P ratio up to 42.9. This is mainly due to a limited source of phosphorous, which is essential for the assimilation of anaerobes and enhances the metabolism processes of 4-nitrophenol. The deficiency of nitrogen (N) and phosphorous (P) source would negatively affect the acid and methane producing bacteria and hence the removal efficiency of 4-nitrophenol (Goodwin et al., 1990; Elreedy et al., 2017). However the sufficient nutrients in the influent regulate the enzymes activities of microbes, overcome biomass washout and the toxicity effect of the phenolic compounds. The optimum N/P ratio for anaerobic degradation of phenolic compounds were 7 (Shen et al., 2006). Supplementation of ammonium sulfate at a concentration of 0.04–0.4 g/L did not significantly affect p-nitrophenol degradation efficiency, and the biotransformation rate in the absence and presence of nitrogen source was almost similar as reported by Samuel et al. (2014).

3.2. *Effect of addition graphene nano-particles on the organics degradation, extracellular polymeric substances and 4-aminophenol recovery in multistage anaerobic reactor*

The presence of 4-NP in the wastewater would have a negative impact on the biodegradation of the organics (Kuşçu and Sponza, 2005). However, the degradation of the organics by methanogens facilitates the

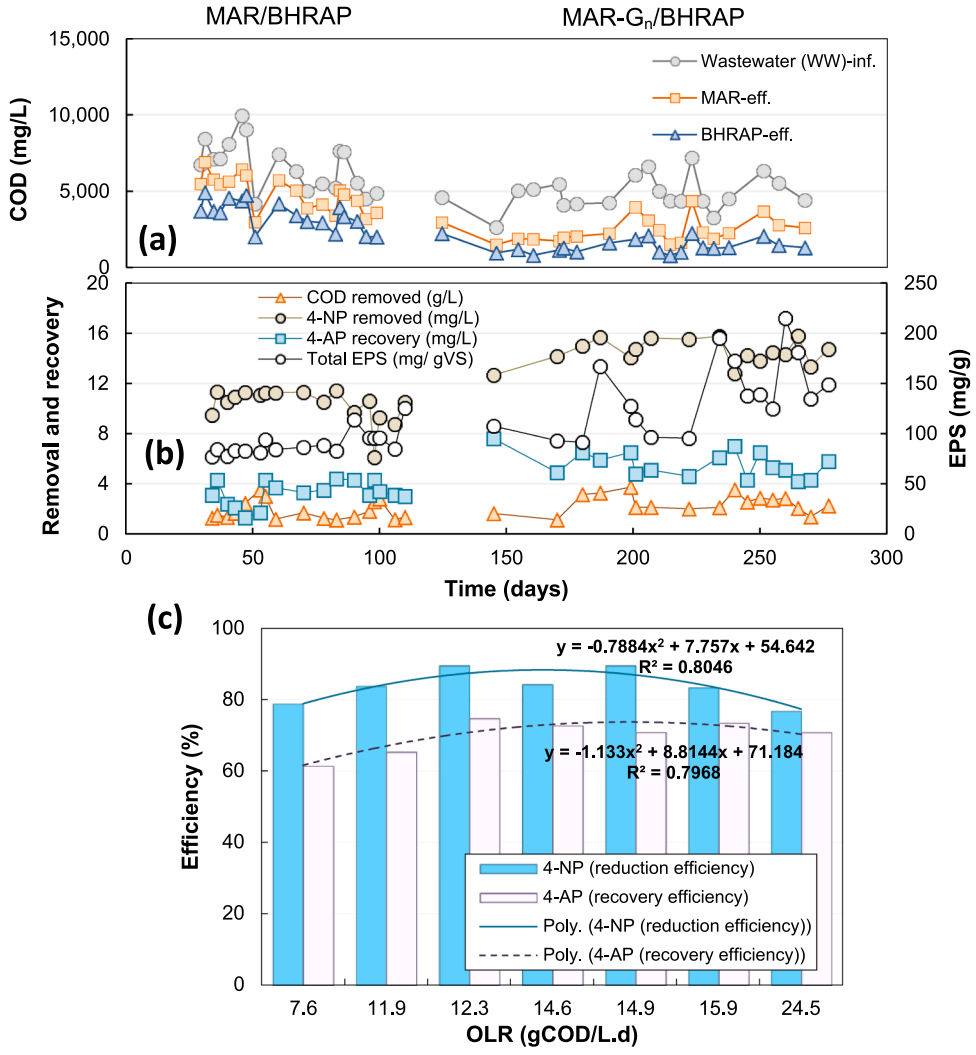

Fig. 4. Effect of addition Graphene nanoparticles on the COD degradation (a), extracellular polymeric substances, and 4-aminophenol recovery (b). (c) Effect of organic loading rate (OLR) on the 4-nitrophenol reduction and 4- amino-phenol recovery.

reduction of 4-NP onto 4-AP. The maximum removal efficiency of COD was 68.3% in the MAR-G_n that was dropped to 36.8% for classical MAR at 4-NP loading rate of 47.9 g/m^3.d, as shown in Fig. 4a. This was linked to the presence of G_n, which enhanced the secretion of extracellular polymeric substances (EPS) in the bulk liquid for organics degradation and reduction of 4-NP (Fig. 4b). The EPS was increased in the MAR-G_n by a value of 33.7%. The G_n regulates the activities of the enzymes, enhances the anaerobic metabolism, and mitigates the inhibition impact of 4-NP. The degradation of 4-NP is supposed to occur in the surrounding medium of the anaerobes where the microorganism secrets the EPS for acceleration of substrate degradation. Moreover, the reduction efficiency of 4-NP and recovery of 4-AP was occurred and accompanied with the COD degradation by bacterial activities (Fig. 4b). The degradation of carbonaceous organic matter would produce electron donors, facilitating the reduction of 4-NP onto 4-AP. The generation of necessary energy mainly accompanies with the COD degradation by anaerobic bacteria for reducing 4-NP (Amezquita-Garcia et al., 2016). The removal efficiency of COD using sequential anaerobic migrating blanket reactor was reduced from 94% to 58% at increasing p-nitrophenol loading rates from 0.96 to 9.63 g/m^3.d (Kuşçu and Sponza, 2005). A higher COD removal efficiency of 79–90% at PNP loading rates of 8.3 g/m^3.d., was reported by Kuşçu, D.T. Sponza (Kuşçu and Sponza, 2005) using anaerobic baffled reactor. Fig. 4c shows the effect of OLR on the reduction efficiency of 4-NP and recovery of 4-AP. The data revealed that the 4-NP reduction efficiency and 4-AP recovery was increased from 78.9% to 89.5% and from 61.4% to 73.5% at increasing the OLR from 7.6 to 15.9 gCOD/L.d., respectively. However, the 4-NP reduction and 4-AP recovery was dropped at OLR exceeding 24.5 gCOD/L.d. Apparently, an increase of organic loading rate (OLR) to some extent would enhance the reduction rate of 4-NP onto 4-AP where the generation of electron donors is sufficient to accomplish the biotransformation process.

3.3. *Anaerobes community degrading 4-nitrophenol*

The microbial community analysis for the sludge immobilized on Gn was conducted to identify the fauna responsible for the degradation of 4-NP. *Bacteroidetes* (23.4%) and *Proteobacteria* (33.4%) were dominant, indicating their contribution for reducing 4-NP into 4-Ap as shown in Fig. 5a. *Caldiserica* (2.2%), *Chloroflexi* (9%), *Firmicutes* (3.8%), *Spirochaetes* (3.0%), *Synergistetes* (1.3%), *Verruccomicrobia* (1.4%) and *Acidobacteria* (2.3%) were also detected in the reactor. Those phylum's were previously reported for organics degradations of the wastewater (Wang et al., 2020), where *Bacteroidetes* and *Proteobacteria* was efficient for COD degradation. Those consortia effectively reduce 4-NP into 4-AP via the transfer of six-electrons. Nitroreductases mainly catalyze this

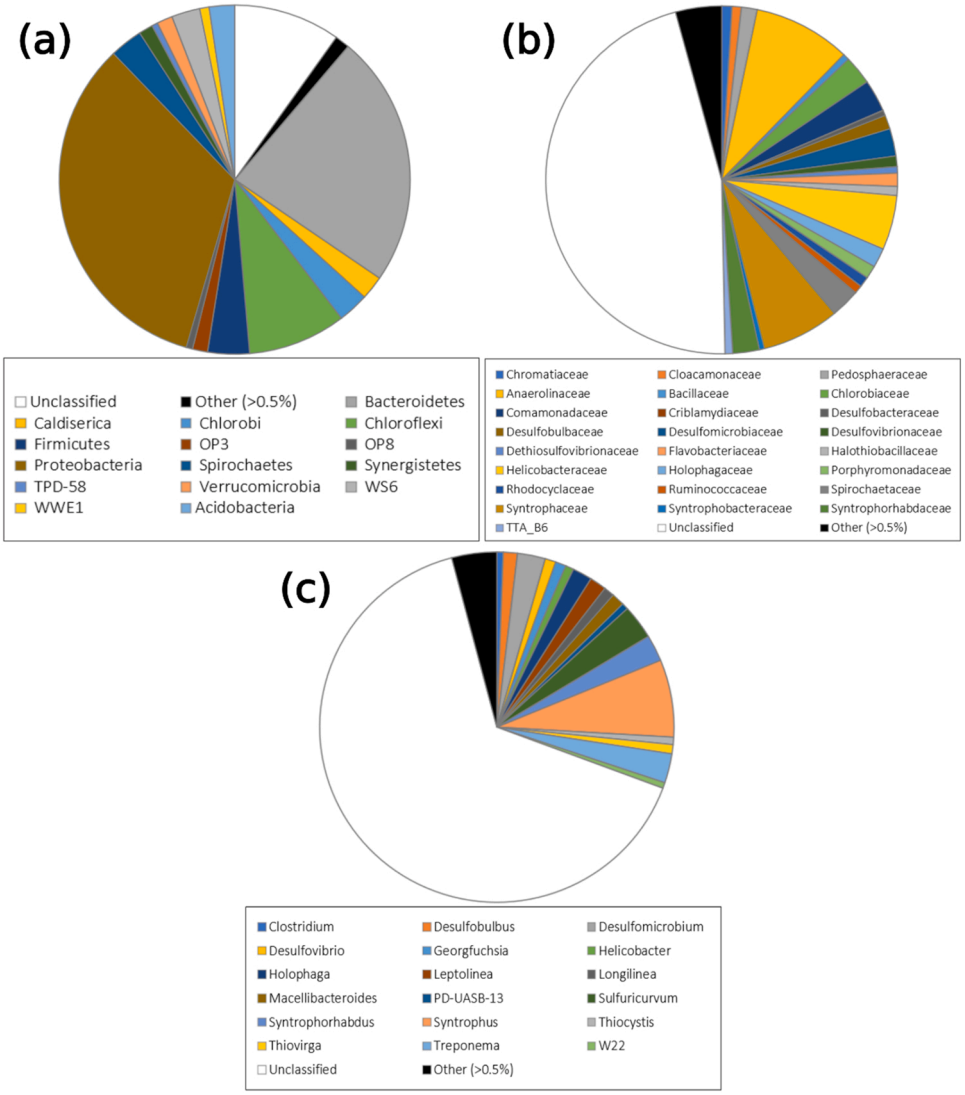

Fig. 5. Bacterial community structure in sludge immobilized on Gn and fed with wastewater rich 4-nitrophenol at the level of phylum (a), family (b) and genus (c) with total OTUs of 14,828.

reaction by the successive supplementation of electron pairs donated by fermentable organics. The 4-NP reduction process is occurred in the presence of synergistic contribution of those consortiums of bacteria. The sludge was very rich with several families mainly, *Syntrophorhabdaceae* (2.43%), *Syntrophaceae* (7.15%), *Spirochaetaceae* (2.7%), *Porphyromonadaceae* (1.2%), *Holophagaceae* (1.8%), *Helicobacteraceae* (5.06%), *Flavobacteriaceae* (1.23%), *Desulfovibrionaceae* (0.59%), *Desulfomicrobiaceae* (2.55%), *Desulfobulbaceae* (1.29%), *Anaerolinaceae* (8.97%), *Pedosphaeraceae* (1.45%) and *Chlorobiaceae* (2.67%) as shown in Fig. 5b. The majority of the genus detected in the sludge was *Treponema* (2.7%), *Syntrophus* (7.1%), *Syntrophorhabdus* (2.4%), *Sulfuricurvum* (3.2%), *Macellibacteroides* (1.2%), *Longilinea* (1%), *Leptolinea* (1.5%) and *Holophaga* (1.8%), *Georgfuchsia* (1%), *Desulfovibrio* (1%), *Desulfomicrobium* (2.5%), *Desulfobulbus* (1.3%) and *Clostridium* (0.6%) as shown in Fig. 5c. The Methanomicrobia, Thermoplasmata, Methanobacteria at class levels, Methanosaetaceae, Methanomassiliicoccaceae, Methanoregulaceae and WSA2 at family levels were detected in the sludge as shown in Table 1. The genus levels of *Methanosaeta* (52.9%), and *Methanosphaerula* (10.9%) were dominant species. *Methanobacterium formicium* and *Desulphatomaculem orientis* completely degraded 3-, 4-NP (Kulkarni and Chaudhari, 2007). *Methanoseta*, *Methanobacterium*, *Leucobacter*, *Geobacter* and *Kosmotoga* were the most dominant UASB module species inoculated with anaerobic granular sludge immobilized on the activated carbon cloths employing for biodegradation of 4-nitrophenol (Buitron et al., 2003). They found methanogenic archaea such as *Methanosaeta* and *Methanobacterium* represented 53% and 18%, respectively. Likely, Graphene oxide enhanced the degradation of nitrophenols and azo-dyes by methanogens and sulfate reducing bacteria due to as electron shuttle for redox conversion (Elreedy et al., 2019).

3.4. Fourier transform infrared spectroscopy (FTIR), scanning electron microscope (SEM) and energy-dispersive X-ray spectroscopy (EDX) of the sludge immobilized on graphene nanoparticles

FT-IR spectra in terms of organic functional groups are characterized for the influent wastewater, MAR-G_n and BHRAP effluents (Fig. 6a). A big absorption band at 3380.5, 3319.24, and 3324.95 cm^{-1} were detected for (O-H) group in the influent wastewater, MAR-G_n, and BHRAP effluents, respectively, due to vibration of H-bonded NH groups. The peaks were reduced due to a drop of the 4-NP and generation of 4-AP in the treated effluents of the MAR-G_n and BHRAP (Réveillé et al., 2003). C-H group was detected at bands of 2919.96 and 2881.51 cm^{-1} in the wastewater influent and disappeared in the MAR-G_n and BHRAP effluents, indicating a low degree of aliphaticity (Réveillé et al., 2003). A small peak was observed at 2121.0 cm^{-1} for a combination of hindered rotation and O-H bending (water) in the influent wastewater and shifted to 2114.51 cm^{-1} and 2115.37 in the MAR-G_n and BHRAP effluents. 1980.11 cm^{-1} was detected in the influent wastewater and disappeared in the MAR-G_n and BHRAP effluents. A small peak was observed in the MAR-G_n effluent at frequency of 1695.46 cm^{-1} due to the presence of amide I and disappeared in BHRAP effluent. Likely a band peak was detected in the effluent of BHRAP at 1636.88 cm^{-1} for CHO stretching of carbonyl group, typical saccharide absorption. However, a sharp, intense absorption band at peaks of 1642.57 and 1639.38 cm^{-1} was observed in the influent wastewater and decreased in the effluent of MAR-G_n due to the vibration of H bonded to C═O carbonyl groups of amide I and disappeared in the effluent of BHRAP due to aromatic and phenolic ring deformations. The peak was stretched at 1348.96 cm^{-1} in the influent wastewater due to the presence of 4-NP, which was disappeared in the effluents of MAR-G_n and BHRAP due to the degradation process. A peak band of 1484.85 cm^{-1} was detected in the effluent of MAR-G_n module, indicating the reduction of 4-NP into 4-AP. The latter was not detected in the BHRAP due to the mixed cultural algal activities (Ramesh et al., 2006). The peak band of 1279.53 cm^{-1} was not detected in the MAR-G_n and BHRAP effluents due to the deformation N–H and phenols degradation. The peak of 1249.54 cm^{-1} (PO_2^- asymmetric and C-N stretching) in the influent was shifted into 1247.24 cm^{-1}(PO_2^- asymmetric-Phosphate I) and 1248.84 cm^{-1} (PO_2^- asymmetric and C-N stretching) in the MAR-G_n and BHRAP effluent, respectively, due to the fluctuations of N-rich compounds particularly amide III groups. A new peak was detected in the effluent of BHRAP at a band of 1158.31 cm^{-1} and a sharp peak was detected at 1085.28 cm^{-1} for PO_2^- symmetric (Phosphate II) for wastewater and significantly reduced in the MAR-G_n and BHRAP effluents at bands of 1085.56 and 1084.81 cm^{-1} respectively. New small peaks were detected in the MAR-G_n and BHRAP effluents at bands of 1157.27 and 1045.06 $cm^{-1,}$ respectively, for C-OH groups of carbohydrates. The peaks of 948.25; 879.12 and 747.66 cm^{-1} was observed in the wastewater due to the presence of aromatic groups (Shon et al., 2004) and was reduced and shifted to 965.63, 877.76, 748.35 cm^{-1} in the MAR-G_n effluent and to 965.41, 877.39, 747.60 cm^{-1} in the BHRAP effluent due to a biodegradation process. The peak of 840.62 cm^{-1} was disappeared in the MAR-G_n and BHRAP effluents due to complete degradation of 4-NP. The peaks of 601.17 and 526.17 cm^{-1} were stretched for aromatic groups in the influent and slightly reduced at bands of 601.66 and 522.10 cm^{-1} in the MAR-G_n effluent and 601.41, 522.21 cm^{-1} in the BHRAP effluent (Shon et al., 2004).

The SEM imaging of sludge immobilized on G_n before treatment (Fig. 6b) and after treatment (Fig. 6c). The graphs displayed a layered structure with a fluffy appearance of the sludge immobilized on G_n after fermentation in MAR-G_n. It also showed high agglomeration of the sheets in the sludge with microbial activities. Microbial cell damage was not detected in SEM analysis of the sludge immobilized on 5.0 mg/L Graphene oxide employing to reduce nitrophenols (Colunga et al., 2015). The elemental analysis of the anaerobes immobilized on G_n before (Fig. 6d) and after treatment (Fig. 6e). The results showed that the sludge's carbon (C) content was increased after treatment from 45.42% to 48.97% due to the growth of the anaerobes. Moreover, the nitrogen (N), oxygen (O), and phosphorous (P) were reduced by values of 21.2%, 8.3%, and 30% after the treatment process. The C/O ratio was increased from 0.92 (before treatment) to 1.1 (after treatment).

Table 1
Methanogenic archaea genera identified in MAR at the end of anaerobic digestion process (Total OTUs = 238).

Class	Family	Genus	Multistage anaerobic reactor
Methanomicrobia	*Methanosaetaceae*	*Methanosaeta*	52.9
Thermoplasmata	*Methanomassiliicoccaceae*	Unclassified	16.8
Methanomicrobia	*Methanosaetaceae*	*Methanosaeta*	15.1
	Methanoregulaceae	*Methanosphaerula*	10.9
Methanobacteria	*WSA2*	Unclassified	4.2

[a]Percentage of sequences affiliated to each genus with respect to the total sequences reliably assigned to methanogenic archaea.

3.5. 4-Aminophenol recovery in baffled high rate algal pond

The 4-AP recovery was accounted for 44.8 ± 1.04% in the treated effluent of the MAR-G_n and 29.8 ± 0.9% in the classical MAR as shown in Fig. 7a. The remaining portions of 4-NP in the anaerobic effluent was removed by mixed culture algae in a baffled high rate algal pond (BHRAP), resulting in only 1.5 ± 0.006 mg/L in the final effluent. The maximum removal efficiency of 4-NP was accounted for 53.2 ± 1.2% with an overall removal efficiency of 91.6 ± 6.3 in the integrated system (MAR-G_n/BHRAP). This is linked to the excessive growth of algal cells in the pond's bulk liquid, resulting in high consumption of dissolved CO_2 and an increase of alkalinity up to 456.6 mg$CaCO_3$/L in the bulk liquid.

Under alkaline conditions, high DO (6.2 ± 0.33 mgO_2/L) and pH levels (9.0 ± 0.21), the opening of the phenolic ring (4-AP) is occurred due to the prevailing oxidative conditions (Fig. 7b). Enhancement of biodegradability of phenolic compounds and break down of phenol rings

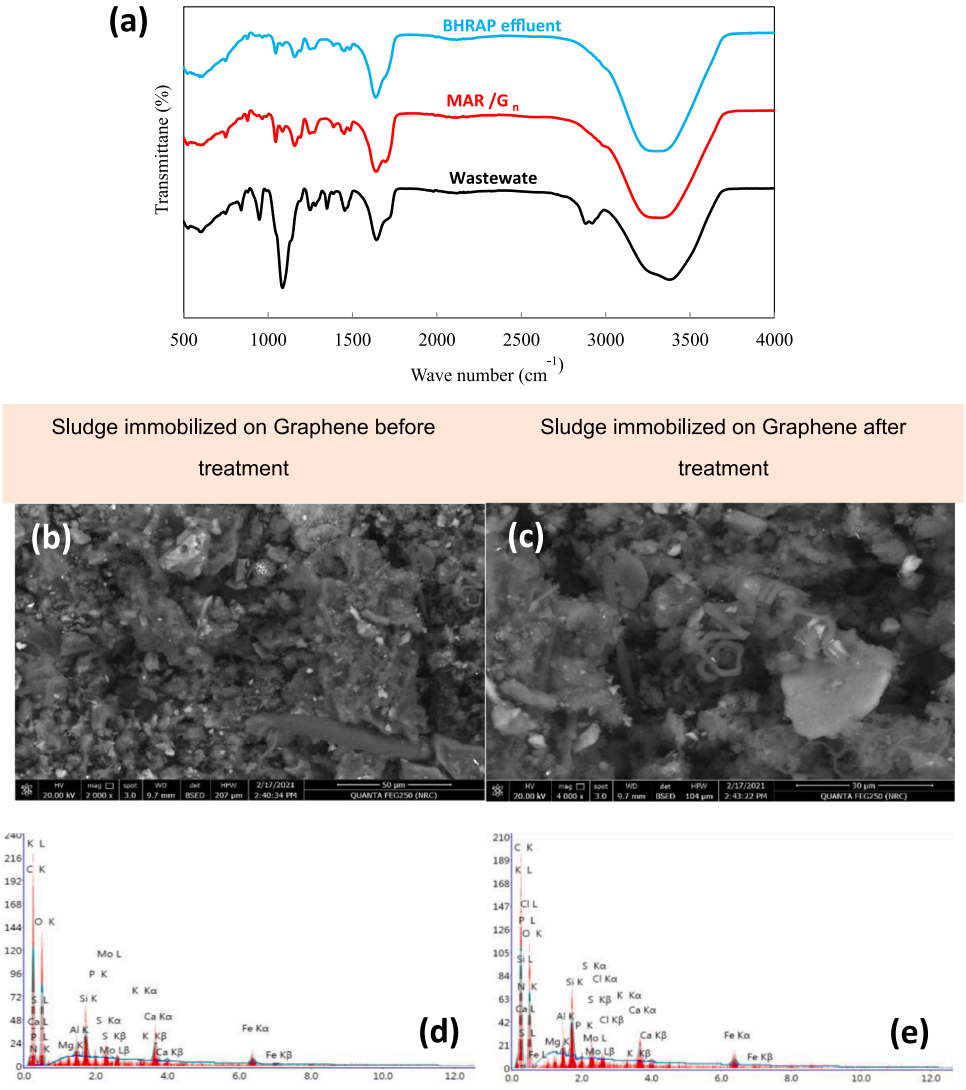

Fig. 6. (a) Fourier transforms infrared spectroscopy (FTIR) of the influent, MAR-G_n effluent and BHRAP-effluent. Scanning Electron Microscope (SEM) images of sludge immobilized on G_n before (b) and after treatment (c), energy-dispersive X-ray spectroscopy (EDX) of sludge immobilized on G_n before (d) and after treatment (e).

under alkaline oxidative conditions was reported by Wang et al. (2020). The HRAP removed 80% of bisphenol A and 32% of bisphenols from wastewater and partially recovered onto biomass (Ağdağ and Sponza, 2005). Similarly, Hirooka et al. (2006) found that mixed culture composed of *A. variabilis* and *A. cylindrica* completely removed 2,4-dinitrophenol. Nevertheless, the less toxicity of 4-AP was removed in the BHRAP unit (96.8 ± 4.0%) due to an oxygenation by photosynthesis process of algal fauna in the BHRAP module at ORP of 127.3 ± 4.1 mV. *Chlamydomonas reinhardtii* and *Anabaena cylindrical* can degrade 2-amino-nitro phenol, and mixed microalgae culture cyanobacteria was efficient for removal of 2,4-dinitrophenol from industrial wastewater (Hirooka et al., 2003b). Apparently, the algae utilized a portion of 4-AP for biomass assimilation, and the existing aerobic consortia have oxidized the organics into carbon dioxide and water in the presence of oxygen (DO = 6.5 mg/L). The microalgae supply oxygen and nutrients to heterotrophic bacteria, and the latter provides the algal cells with carbon dioxide from the degradation of 4-AP for growth. The remaining residual COD in the effluents of MAR-G_n and MAR was partially removed in the BHRAP by bacterial/algal interaction, resulting in removal efficiency of 42 ± 9.5% and 30.3 ± 6.2%, respectively, as shown in Fig. 4a. However, the COD effluent quality is still not complying for discharge due to the presence of some algal cells in the treated effluent, thus needs further treatment process. The removal of organic matter in the algal pond system is mainly due to the interaction between algal cells and aerobic bacteria. The algal cells produce oxygen for bacterial activity, and the aerobes generate CO_2 to grow algal biomass. The organics removal in the algal pond was due to aerobic heterotrophs in the reactor (Wang et al., 2020). Values of 57–59% removed the COD due to the dual interaction between microalgal cells and heterotrophs at DO of 6 mg/L (Rodrigues de Assis et al., 2020). An anaerobic/aerobic system achieved the higher removal efficiency of the COD (97%) at a lower loading rate of 3.85 g p-NP/m^3.d (Ağdağ and Sponza, 2005).

The nutrients (P&N) play a big role in biomass synthesis and biodegradation efficiency. The removal efficiency of 4-NP would be affected at different NH_4/P ratios, particularly in the BHRAP where the buildup of algal cells are mainly depends on the nutrient supplementation in the feed. The effect of NH_4/P ratio on the utilization of the 4-AP, which resulted from anaerobic transformation process, was significant in BHRAP, as shown in Fig. 7c. The removal efficiency of 4-AP was complete (100%) at NH_4/P ratios of 12.9–26.3, which was deteriorated and dropped to 83.3% at the NH_4/P ratio of 50.5 resulting in a quite low value of 0.9 mg4-AP/L in the final effluent. C. pyrenoidosa achieved removal efficiency of 95% of the phenol with nutrients supplementations (Stephen and Ayalur, 2017). The major portion of NH_4-N and

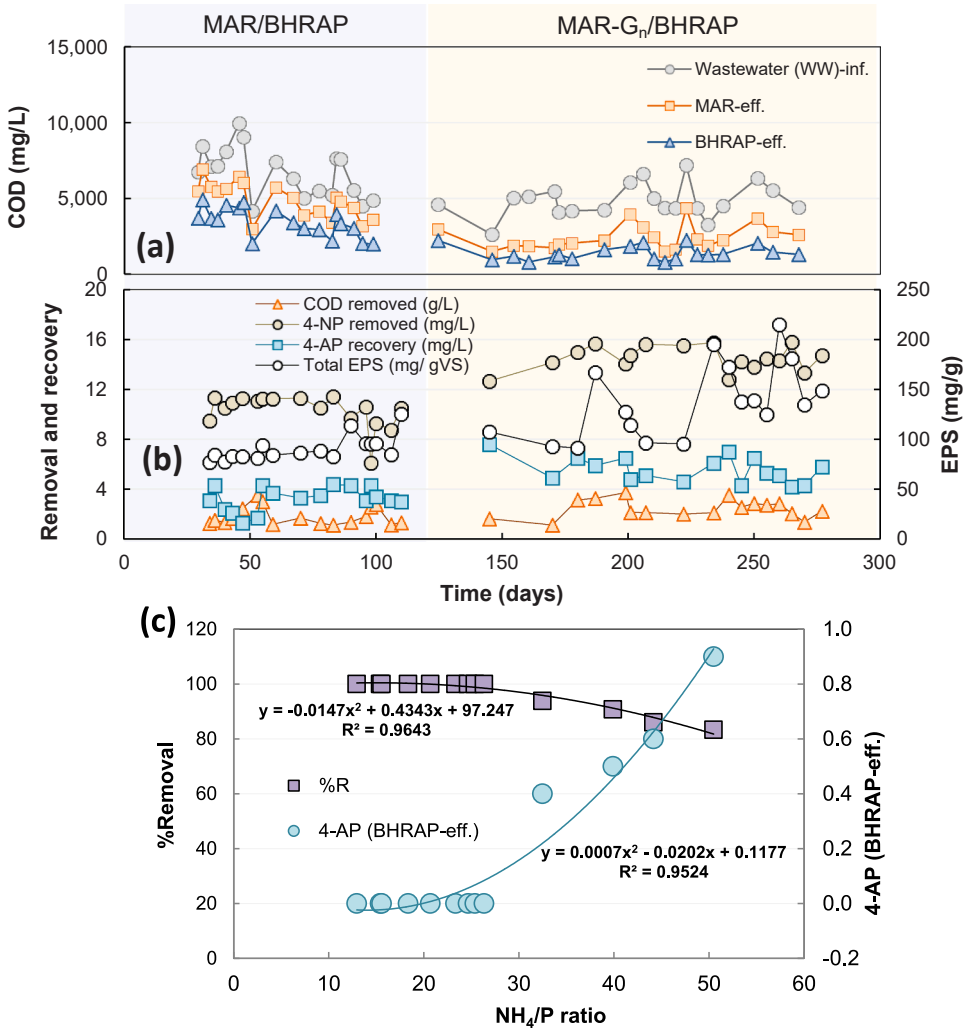

Fig. 7. 4-Aminophenol recovery (a), pH values and dissolved oxygen (b) in the integrated system. (c) Effect of NH_4/P ratio on the 4-aminophenol recovery.

phosphorous is mainly utilized for bio-algal growth, which affects the biodegradation of 4-AP. Nevertheless, at an alkaline pH value of 9.0, the volatilization of NH_3 and orthophosphate precipitation occurs (Muñoz and Guieysse, 2006). As a result, 78% of NH_4-N and 16% of TP was removed by a biofilm algal pond system treating wastewater (Rodrigues de Assis et al., 2020).

Further reduction of the IC_{50} up to 100 ± 6.8 μg Gallic acid equivalent/mL was observed in the BHRAP due to a highly reduction of 4-NP in the in the effluent of anaerobic module as shown in Fig. 3b. Kulkarni et al., (Kulkarni and Chaudhari, 2006) found that the toxicity increased at decreasing pH values, and subsequently, conversion was fully inhibited in acidic pH. Alkaline pH highly reduced the toxicity and accelerated the metabolism process in the BHRAP. The toxicity was less at an alkaline pH value of 9.0 ± 0.19 in the BHRAP reactor due to a release of carbon dioxide the reaction medium (Fig. 7b).

3.6. Algal species characteristics and community analysis

Characterization of algal cells and algal species are presented in Fig. 8a and b. The algal cells' lipids, carbohydrates, and proteins amounted to 7.6 ± 0.05, 23.1 ± 0.9, and 41.5 ± 1.4%. Likely, 32% of protein, 32% of carbohydrates, and 18% of total lipids were observed for algal cells treating wastewater industry (Rodrigues de Assis et al., 2020). The algal cells contained total phenolic compounds of 180 ± 8.7 μg/g, total antioxidants of 14.4 ± 0.67 μg/g, total flavonoids of 36 ± 0.9 μg CE /g, Carotenoids of 11.21 ± 0.9 μg/g and anthocyanin of 0.12 ± 0.009 mg/g are shown in Fig. 8a. This indicates that partial removals of phenolic compounds were taken place due to the up-take process. The Chlorophyll a (Chl-a) and (Chl-b) amounted to 216.28 ± 4.9 and 222.37 ± 7.9 μg/g biomass, respectively, positively affecting COD and 4-AP removal efficiency. The algal cells during the irradiation process are gradually destroyed resulting pigments such as chlorophyll-a, chlorophyll-b and carotenoids in the bulk liquids. Those pigments could play a vital role for adsorption and degradation of 4-NP and 4-AP as reported by Wang et al. (2007). The COD had a good correlation with Chl-a and algal growth activities in aerated membrane algal system (Buitrón et al., 2005). Fig. 8b shows the dominant phylum of algal species in the BHRAP treating the MAR-G_n effluent. *Chlorophyta cells* in terms of *Chlorella vulgaris* (5.684×10^3 cells /mL), *Scenedesmus obliquus* (9.71×10^3 cells/mL), *Scenedesmus quadricauda* (1.544×10^3 cells/mL), and *Ulothrix subtilissima* (4.268×10^3 cells/mL) were observed in the BHRAP. Those algal species were very effective for the removal of 4-NP and 4-AP (Figs. 3a and 7a). The removal of contaminants is mainly based on the algal-bacteria interaction and adsorption onto the dead algal cells (Xu et al., 2015). > 60% *Chlorella vulgaris* was the dominant species in the algal pond system treating wastewater (Rodrigues de Assis et al., 2020). *Chlorella vulgaris* was efficient for degradation and adsorption of phenolic compounds (Tamer et al., 2006) and *Chlorella pyrenoidosa* was reported for degradation of nitro-aromatic compounds (4-nitroaniline and 4-NP). 4-NP is normally biodegraded by microbes via two mechanism pathways i.e. 4-NP converted into maleylacetate through hydroquinone or transformed into 1,2,4-benzenetriol

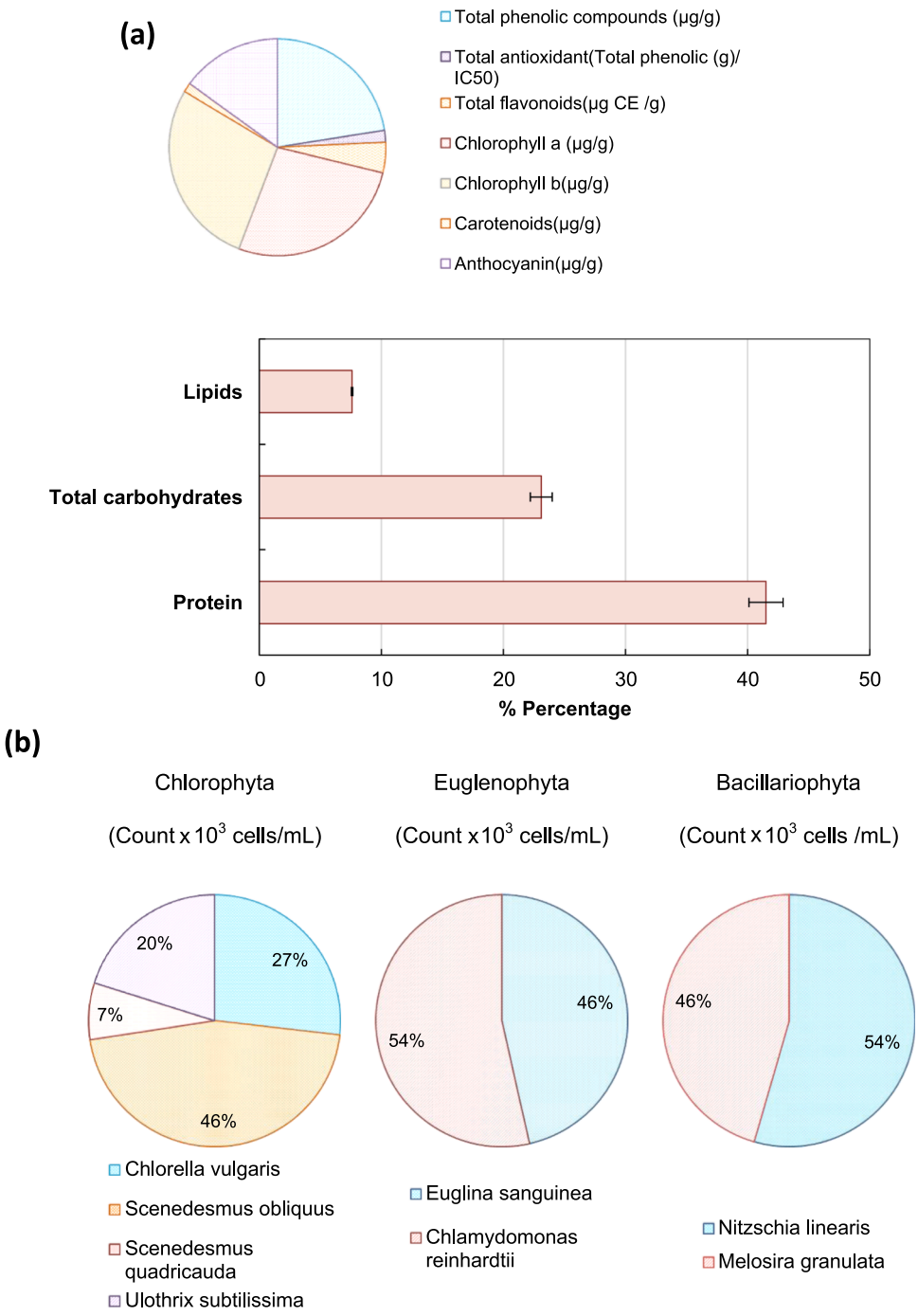

Fig. 8. Algal cells characterization (a) and algal species (b) in the baffled high rate algal pond (BHRAP) system.

via 4-nitrocatechol. Dioxygenase is utilized in the presence of oxygen for hydroxylation of phenol ring in algal treatment system (Xu et al., 2015). *Euglenophyta* cells was quite high in the pond system i.e. *Euglina sanguinea* (10.803×10^3 cells/mL) and *Chlamydomonas reinhardtii* (12.438×10^3 cells/mL). *Bacillariophyta* phylum was the lowest (1.327×10^3 cells/mL for *Nitzschia linearis* and 1.109×10^3 cells/mL for *Melosira granulate*) as shown in Fig. 8b. Cyanobacteria and eukaryotic microalgae i.e *Chlorella* sp., *Scenedesmus* sp., *Selenastrum capricornutum, Tetraselmis marina, Nostoc punctiforme and Oscillatoria animalis* is efficient for biotransformations of phenolic compounds as reported by Lika et al., (Lika and Papadakis, 2009). *Chlorella pyrenoidosa* based on 18SrRNA sequence analysis (KX686118) reduced the phenol concentration by a value of 97.4% from phenolic wastewater of a coal gasification plant at an initial concentration of 0.8 g of phenol/L (Dayana Priyadharshini and Bakthavatsalam, 2016, 2019). 2,4-dimethyl phenol was metabolized by *Chlorella* sp. at initial concentration of 1000 mg/L (Semple and Cain, 1996) and de-chlorinated by 2-chlorophenol at a concentration of 200 mg/L. *Scenedesmus* sp. biodegraded wastewater containing 2,4-dinitrophenol at concentration of 190 mg/L.

4. Conclusions

The integrated system composed of the multistage anaerobic reactor (MAR) and baffled high rate algal pond (BHRAP) system completely eliminated 4-nitrophenol from wastewater industry at a loading rate of 47.9 g/m^3.d. The FTIR results showed the band peak was stretched at

1348.96 cm^{-1} in the influent wastewater due to the presence of 4-NP, which was disappeared in the effluents of MAR-G_n and BHRAP due to the biodegradation process. Immobilization of anaerobes on G_n promoted the secretion of extracellular polymeric substances and increased the reduction efficiency of 4-NP into 4-AP. Graphene nanoparticles (G_n) was served as electron shuttle enhancing the reduction efficiency of 4-NP, and methanogens utilized the VFAs as electron donor. Moreover, G_n promoted the growth of methanogens i.e., *Methanosaeta* and *Methanobacterium*. The algal cells contained total phenolic compounds of 180 ± 8.7 μg/g, and total flavonoids of 36 ± 0.9 μg CE /g, indicating that partially removals of phenolic compounds were taken place due to the up-take process. It is recommended to apply such an integrated system for detoxification wastewater industry. However, further research is needed for enzymes responsible for degradation of 4- nitrophenol and metabolite-by products reaction pathway.

CRediT authorship contribution statement

Ahmed Tawfik: Conceptualization, Funding acquisition, Writing, Investigation, Review & editing. **Khaled Hasanan**: Writing, Investigation, Review & editing. **Mahmoud Abdullah**: Methodology, Formal analysis. **Omnia A. Badr**: Writing, Methodology, Formal analysis. **Hanem M. Awad**: Methodology, Formal analysis. **Mohamed Elsamadony**: Methodology, Formal analysis. **Ali El-Dissouky**: Methodology, Formal analysis. **Muhammad Abdul Qyyum**: Conceptualization, Writing, Investigation, Review & editing. **Abdul-Sattar Nizami**: Review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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