

Article

# Magnesium Aminoclay-Fe<sub>3</sub>O<sub>4</sub> (MgAC-Fe<sub>3</sub>O<sub>4</sub>) Hybrid Composites for Harvesting of Mixed Microalgae

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Received: 5 April 2018; Accepted: 25 May 2018; Published: 26 May 2018



**Abstract:** In this paper, we describe the synthesis of magnesium aminoclay-iron oxide (MgAC-Fe<sub>3</sub>O<sub>4</sub>) hybrid composites for microalgae-harvesting application. MgAC-templated Fe<sub>3</sub>O<sub>4</sub> nanoparticles (NPs) were synthesized in different ratios of MgAC and Fe<sub>3</sub>O<sub>4</sub> NPs. The uniform distribution of Fe<sub>3</sub>O<sub>4</sub> NPs in the MgAC matrix was confirmed by transmission electron microscopy (TEM). According to obtained X-ray diffraction (XRD) patterns, increased MgAC loading leads to decreased intensity of the composites' (311) plane of Fe<sub>3</sub>O<sub>4</sub> NPs. For harvesting of *Chlorella* sp. KR-1, *Scenedesmus obliquus* and mixed microalgae (*Chlorella* sp. KR-1/ *Scenedesmus obliquus*), the optimal pH was 4.0. At higher pHs, the microalgae-harvesting efficiencies fell. Sample #1, which had the highest MgAC concentration, showed the most stability: the harvesting efficiencies for *Chlorella* sp. KR-1, *Scenedesmus obliquus*, and mixed microalgae were reduced only to ~50% at pH = 10.0. The electrostatic interaction between MgAC and the Fe<sub>3</sub>O<sub>4</sub> NPs in the hybrid samples by microalgae, as confirmed by zeta potential measurements, were attributed to the harvesting mechanisms. Moreover, the zeta potentials of the MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid composites were reduced as pH was increased, thus diminishing the microalgae-harvesting efficiencies.

**Keywords:** magnesium aminoclay (MgAC); magnetite; *Chlorella* sp. KR-1; *Scenedesmus obliquus*; microalgae harvesting; biorefinement

## 1. Introduction

Microalgae convert sunlight and carbon dioxide to essential materials such as biofuels, pharmaceutical bioactives, and fertilizers [1–5]. Indeed, lipids obtained from microalgae promise to replace fossil fuels [6]. However, such potential remains limited by the costs of microalgae cultivation, harvesting, dewatering, lipid extraction, and conversion to biodiesel [6].

Among the various nanomaterials that could be applied for microalgae cultivation and harvesting, magnesium aminoclay (MgAC) is a potential candidate [7]. MgAC, which was first introduced

by Mann et al. (1997) [8], is attractive due to its propylamine functionalities, structures, as well as high dispersity in water [9,10]. We have already reported on several studies using MgAC for microalgae-cultivation improvement [11], harvesting [12,13], recovery [14], and also lipids extraction from microalgae biomass [15].

MgAC significantly enhances microalgae-harvesting efficiencies [6] while is not effected by inhibition factors such as humic acid [16]. For example, in one study conducted several years ago, 100% of *Chlorella* sp. KR-1 (1.7 g/L microalgae feedstocks) was harvested 30 min after injection of MgAC at a concentration above 0.6 g/L while maintaining the neutral pH [12]. Farooq et al. (2013) achieved flocculation of microalgae by electrostatic interaction with high-positive-surface-charged MgAC, as confirmed by the obtained zeta potentials [12]. Ji et al. (2016) more recently utilized CeAC with MgAC in a two-ACs-mixture system for cyanobacteria harvesting. The harvesting efficiency of the mixed aminoclays reached 100% within 1 h. Moreover, the mixture loading was 10 times lower than for single-aminoclay treatment [13].

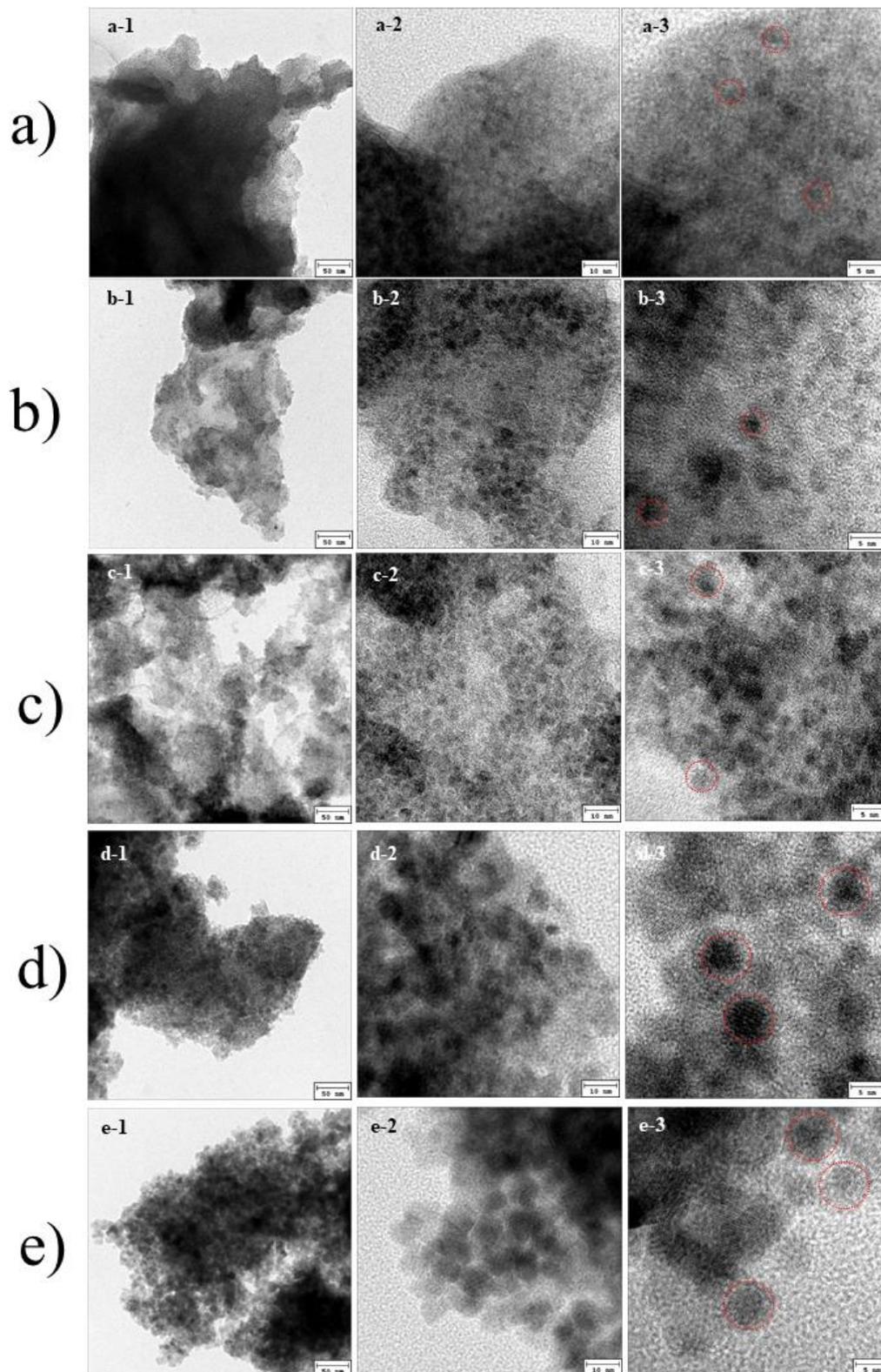
MgAC has the additional potential to be incorporated with other nanomaterials for microalgae-harvesting improvement. To overcome the limitation of MgAC in reusability, we have performed MgAC-templated zero-valent iron (MgAC-nZVI) synthesis for harvesting of *Chlorella* sp. KR-1. This composite showed a high positive-surface charge (+40 mV) and ferromagnetic properties (30 emu/g). On the laboratory-scale, MgAC-nZVI enabled harvesting of *Chlorella* sp. KR-1 (concentration: 20 g/L) within 3 min under a magnetic field, the microalgae-harvesting efficiencies having reached ~100% [14]. We also have already conjugated MgAC with TiO<sub>2</sub> for simultaneous harvesting and wet-disruption of *Chlorella* sp. KR-1 [17]. MgAC played a key role in microalgae flocculation, while TiO<sub>2</sub> contributed to direct cell disruption under UV-light irradiation (365 nm). Within 10 min, the injection of MgAC into the microalgae feedstocks at a concentration of 1.5 g/L effected an ~85% harvesting efficiency; subsequently, the harvested wet-microalgae biomass was irradiated under UV light for 4 h, thus enabling ~95% cell-disruption efficiencies [17].

A previous study indicated the potential of combination of MgAC with metal oxide nanoparticles for microalgae harvesting purpose [17]. In the present study, we utilized magnetic Fe<sub>3</sub>O<sub>4</sub> NPs as another candidate for templating with MgAC and, thereby, harvesting. Fe<sub>3</sub>O<sub>4</sub> NPs have been used previously for harvesting of *Botryococcus braunii* and *Chlorella ellipsoidea* [18]. The microalgae-harvesting mechanism has been attributed to the electrostatic interaction between Fe<sub>3</sub>O<sub>4</sub> NPs and microalgae. Meanwhile, the development of in-situ magnetic separation technology provide the potential for further microalgae-harvesting improvement [18].

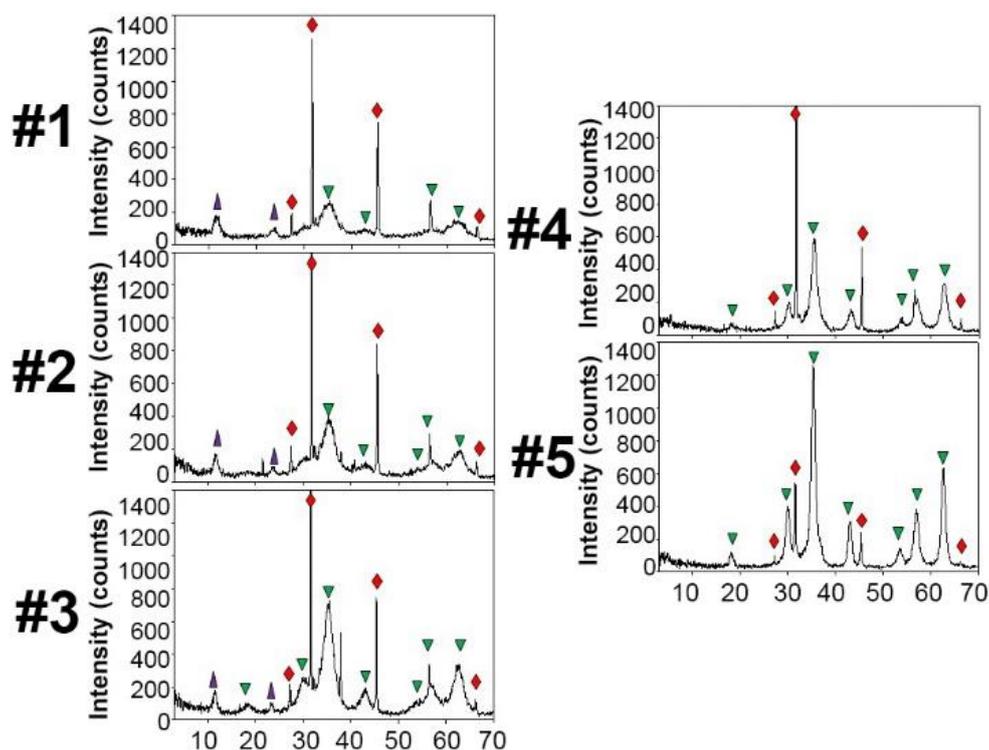
## 2. Results

### 2.1. Characterization of MgAC-Fe<sub>3</sub>O<sub>4</sub> Hybrid Composites

On transmission electron microscopy (TEM) images (Figure 1), the Fe<sub>3</sub>O<sub>4</sub> NPs had approximately crystalline sizes of ~3.50 nm (sample #1), ~3.57 nm (sample #2), ~4.28 nm (sample #3), ~6.42 nm (sample #4), and ~7.14 nm (sample #5, only Fe<sub>3</sub>O<sub>4</sub> NPs). The Fe<sub>3</sub>O<sub>4</sub> NPs were distributed uniformly in the MgAC matrix [17,19,20]. According to the X-ray diffraction (XRD) patterns (Figure 2), all of the as-prepared samples had seven main peaks, at 18°, 29.98°, 35.3°, 42.9°, 54°, 56.37°, and 62°, belonging to (002), (200), (311), (400), (422), (511), and (440) of Fe<sub>3</sub>O<sub>4</sub> magnetite (JCPDS-00-019-0629; JCPDS: Joint Committee on Powder Diffraction Standard) [21], respectively. Meanwhile, from sample #1 to sample #3, there were additional peaks at 10.81° and 22.33° belonging to (002) and (020,110) of MgAC [22], respectively. In the sample #4, the peaks of MgAC were disappeared due to its low ratios in the hybrid composites (Tables 1 and 2). It was apparent that in the hybrid composite, from sample #1 to sample #3, the increase of the MgAC loading concentration resulted in the decrease of the intensity of the (311) plane of Fe<sub>3</sub>O<sub>4</sub>. The appearance of NaCl halite (JCPDS-00-005-0628) indicated remaining NaCl, even after washing.



**Figure 1.** Transmission electron microscopy (TEM) images of (a) sample #1; (b) sample #2; (c) sample #3; (d) sample #4; and (e) sample #5. The crystalline sizes of the Fe<sub>3</sub>O<sub>4</sub> nanoparticles (NPs) are shown within the dotted red circles.



**Figure 2.** Powder X-ray diffraction (PXRD) patterns of samples #1–5. ▲ (MgAC), ◆ (NaCl halite, JCPDS-00-005-0628; JCPDS: Joint Committee on Power Diffraction Standard), and ▼ (Fe<sub>3</sub>O<sub>4</sub> magnetite, JCPDS-00-019-0629).

**Table 1.** Elemental compositions (wt. %) of as-prepared samples by X-ray fluorescence (XRF) spectrometry.

Sample	#1	#2	#3	#4	#5
Fe (%)	72.6	77.7	82.8	86.0	94.7
Mg (%)	5.3	4.6	3.8	2.0	0.2
Si (%)	2.2	1.5	1.3	1.7	0
Cl (%)	19.9	16.2	12.0	9.9	5.1

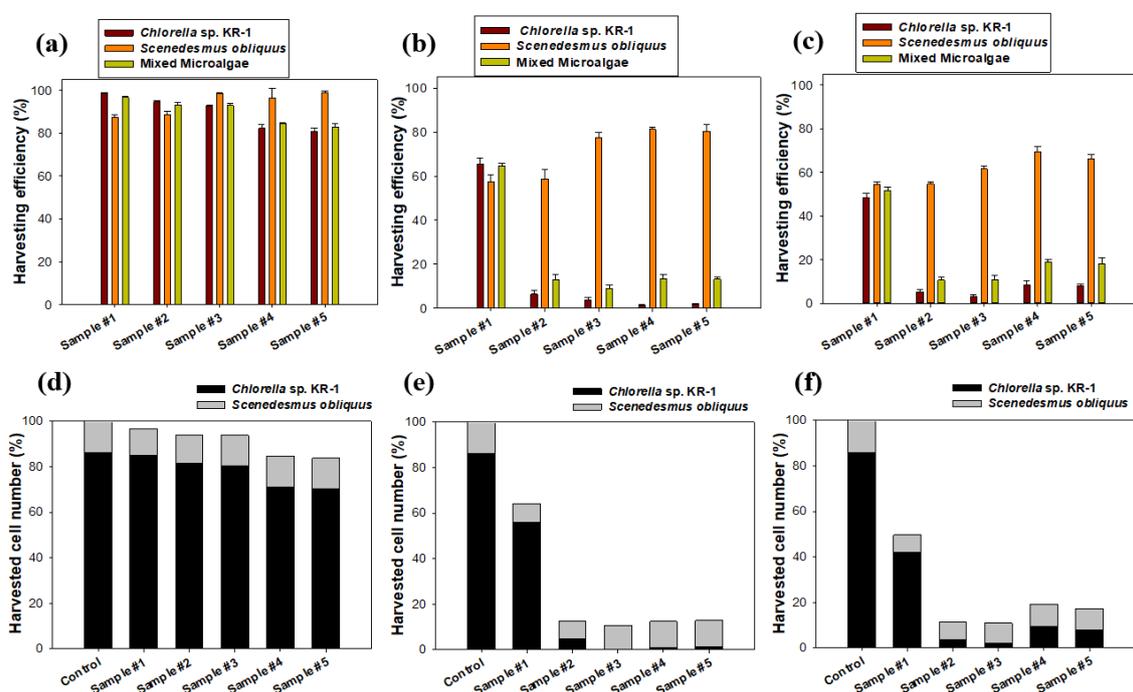
**Table 2.** Formulations of magnesium aminoclay (MgAC) and iron precursors in 90 mL volumes for preparation of MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid composites.

Reactants	Sample #1	Sample #2	Sample #3	Sample #4	Sample #5
MgAC (g/40 mL)	0.80	0.40	0.08	0.04	0
Nanosized Fe <sub>3</sub> O <sub>4</sub> by precipitation method (40 mL)	[1.9 g FeCl <sub>3</sub> •6H <sub>2</sub> O ⊕ 0.7 g FeCl <sub>2</sub> •4H <sub>2</sub> O]				
	10 mL NaOH (10 M)				

## 2.2. Microalgae-Harvesting Efficiencies of MgAC-Fe<sub>3</sub>O<sub>4</sub> Hybrid Composites

The optimal microalgae-harvesting pH for all of the MgAC-Fe<sub>3</sub>O<sub>4</sub> samples (#1–5) was 4.0, at which the harvesting efficiencies were better than 80% for *Chlorella* sp. KR-1, *Scenedesmus obliquus*, and mixed microalgae (*Chlorella* sp. KR-1 and *Scenedesmus obliquus* were mixed in a 1:1 ratio by volume) (Figure 3a). Higher pHs resulted in diminished microalgae-harvesting results (Figure 3). For samples #2, #3, #4, and #5, the harvesting efficiencies for *Chlorella* sp. KR-1 and mixed microalgae were below 20% at pH = 7.0 and 10.0, while those for *Scenedesmus obliquus* were 60–80% at pH = 7.0 and 55–75% at

pH = 10.0, respectively. In the mixed-microalgae harvesting, *Chlorella* sp. KR-1 was more affected by the pH condition of the environment (Figure 3d–f) than was *Scenedesmus obliquus*.



**Figure 3.** Microalgae-harvesting efficiencies (%), top) of respective *Chlorella* sp. KR-1, *Scenedesmus obliquus* and mixed microalgae (*Chlorella* sp. KR-1 and *Scenedesmus obliquus* were mixed in a 1:1 ratio by volume) by samples #1 - #5 at pH 4.0 (a); pH 7.0 (b); and pH 10.0 (c) and corresponding proportions (%), bottom) of respective *Chlorella* sp. KR-1 and *Scenedesmus obliquus* in mixed microalgae by samples #1–#5 at pH 4.0 (d); pH 7.0 (e); and pH 10.0 (f). Control is proportions of *Chlorella* sp. KR-1 and *Scenedesmus obliquus* in initial media.

Sample #1 showed the most stability, which harvesting efficiencies for single *Chlorella* sp. KR-1, *Scenedesmus obliquus*, and mixed microalgae were reduced from >80% at pH = 4 to around 60% at pH = 7.0 and 50% at pH = 10.0 (Figure 3). The greater stability of sample #1 under the high-pH condition was owed to its highest concentration of MgAC (Table 1), which is less affected by high pH [12].

The mechanism of microalgae harvesting is the electrostatic interaction between MgAC and  $\text{Fe}_3\text{O}_4$  NPs in the hybrid samples (which have a positively charged surface) with microalgae (which have a negatively charged surface) [13,18]. The decreased harvesting efficiency of the MgAC- $\text{Fe}_3\text{O}_4$  hybrid composites under the high-pH condition could be confirmed by the decreased zeta potential. For example, the zeta potential of sample #4 decreased from +30 (mV) at pH = 4.0 to 0 (mV) at pH = 8.0 and −4 (mV) at pH = 10, while that of sample #1, which showed the best stability, decreased from +30 (mV) at pH = 4.0 to +20 at pH = 8.0 and +8.5 (mV) at pH = 10.0 (Figure 4). Farooq et al. (2013) explained that the degree of harvesting efficiency of aminoclay is attributable to its high positive zeta potential [12].

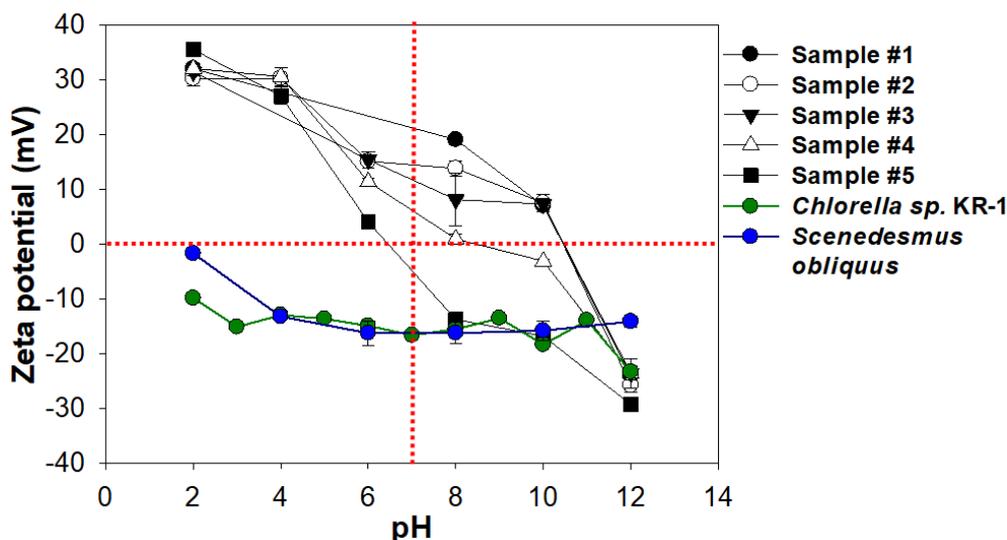


Figure 4. Zeta potentials of samples #1–5, *Chlorella sp. KR-1* and *Scenedesmus obliquus* according to pH.

### 3. Discussion

Due to the difficulty of single microalgae cultivation on a large scale because it goes against natural ecological tendencies, mixed microalgae cultivation could be alternative approach due to its potential in both of increase biomass yields, and the crop protection requirements for commercial applications [23]. Besides cultivation, the harvesting process of mixed microalgae also needs to be studied. In this paper, we chose mixed microalgae (*Chlorella sp. KR-1* and *Scenedesmus obliquus* were mixed in a 1:1 ratio by volume) for testing the harvesting efficiency of MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid nanocomposites.

Fe<sub>3</sub>O<sub>4</sub> NPs, which is well-known by its microalgae harvesting ability due to electrostatic attraction and magnetic properties for recovery after harvesting process [18]. However, the common disadvantage of Fe<sub>3</sub>O<sub>4</sub> NPs is its decrease of surface charge at neutral and alkaline pH conditions [18]. Gao et al. (2009) developed montmorillonite-Cu(II)/Fe(III) for the harvesting of *Microcystis aeruginosa*, but the harvesting efficiencies still decreased at high pH values [24]. Ge et al. (2015) overcame the limitation of Fe<sub>3</sub>O<sub>4</sub> NPs by coating polyethylenimine (PEI) on the surface of Fe<sub>3</sub>O<sub>4</sub> NPs. This nanocomposite still remained the harvesting efficiencies toward *Scenedesmus dimorphus* around 80% at pH of 7.0 [25]. But as indicated from our results, the harvesting efficiencies of *Scenedesmus* species by Fe<sub>3</sub>O<sub>4</sub> NPs just slightly reduced (harvesting efficiency of *Scenedesmus obliquus* by Fe<sub>3</sub>O<sub>4</sub> NPs and MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid nanocomposites was around ~60–80% at pH of 7.0 (Figure 3b)) by the increase of pH value compared to *Chlorella* species (*Chlorella sp. KR-1* in this paper).

MgAC has been showed its potential for microalgae harvesting in previous reports [12,13]. However, due to its high dispersion in water [6], it could not be recycled after harvesting process. In this paper, we synthesized MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid nanocomposites to take advantages of both nanomaterials in microalgae harvesting while reducing their drawbacks. Synthesized MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid nanocomposites easily prepared by the sol-gel method were applied for microalgae harvesting. Fe<sub>3</sub>O<sub>4</sub> NPs were found to be of uniform distribution in the MgAC matrix; their crystalline sizes, moreover, were reduced by the presence of MgAC. The mechanism of this phenomenon should be elucidated in future works. The optimal pH for the harvesting of *Chlorella sp. KR-1*, *Scenedesmus obliquus*, and mixed microalgae was 4.0, beyond which level, the microalgae-harvesting efficiencies were reduced. The harvesting mechanism, as confirmed by zeta potential measurements, was the electrostatic interaction between MgAC and the Fe<sub>3</sub>O<sub>4</sub> NPs in the hybrid samples with microalgae. The zeta potentials of the MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid composites were reduced as pH was increased, and thereby, the harvesting efficiencies were diminished. The combination of MgAC with Fe<sub>3</sub>O<sub>4</sub> NPs improved the harvesting efficiencies but still not completely overcome the limitation of Fe<sub>3</sub>O<sub>4</sub> NPs

at high pH condition. The MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid composites require improvement in order to overcome the problem of reduced harvesting efficiency at high pH before testing their magnetic properties for reusability in future studies.

In conclusion, meanwhile the presence of MgAC in MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid nanocomposite could improve the microalgae harvesting of single and mixed microalgae, this strategy still not completely overcome the limitation of Fe<sub>3</sub>O<sub>4</sub> NPs at high pH values. We also indicated that between *Chlorella* sp. KR-1 and *Scenedesmus obliquus*, the harvesting of Fe<sub>3</sub>O<sub>4</sub> NPs and MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid nanocomposite towards *Scenedesmus obliquus* seems less affected by pH value of environment.

## 4. Materials and Methods

### 4.1. Materials

(3-aminopropyl)triethoxysilane (APTES; ≥98%, 221.37 g/mol), iron(II) chloride tetrahydrate (FeCl<sub>2</sub>•4H<sub>2</sub>O, 98%), and iron(III) chloride hexahydrate (FeCl<sub>3</sub>•6H<sub>2</sub>O, 97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Magnesium chloride hexahydrate (MgCl<sub>2</sub>•6H<sub>2</sub>O; 98%) was obtained from Junsei Chemical (Tokyo, Japan). Ethanol (18 L, 95%) was purchased from Samchun Pure Chemicals (Pyungtack, Korea). NaOH (pellet, >97%) was obtained from Daejung Chemicals & Metals (Siheung, Korea). Distilled-deionized water (DI; resistance: >18 mΩ) was employed in all of the experiments.

### 4.2. Preparation of Magnesium Aminoclay (MgAC)

A total of 1.68 g of MgCl<sub>2</sub>•6H<sub>2</sub>O was diluted in 40 mL of ethanol (95%), mixed with 2.6 mL APTES, and continuously stirred for 8 h. The resulting white-solid was centrifuged and washed three times by ethanol and dried at 60 °C, after which it was ground into powder [26].

### 4.3. Preparation of Aminoclay-Fe<sub>3</sub>O<sub>4</sub> Hybrid Composites

[1.9 g FeCl<sub>3</sub>•6H<sub>2</sub>O ⊕ 0.7 g FeCl<sub>2</sub>•4H<sub>2</sub>O] was dissolved in 40 mL of DI water and continuously stirred for 30 min before 10 mL of NaOH 10 M was added; stirring thereafter was continued overnight (12 h). MgAC in amounts of 0.80, 0.40, 0.08, 0.04, and 0 g was dissolved separately in 40 mL of DI water and then mixed with 40 mL of [1.9 g FeCl<sub>3</sub>•6H<sub>2</sub>O ⊕ 0.7 g FeCl<sub>2</sub>•4H<sub>2</sub>O] solution, again with stirring overnight (12 h). The mixture was then centrifuged, washed with water (3 × 50 mL), dried at 60 °C, and ground to form MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid composites (Table 2).

### 4.4. Characterization of MgAC-Fe<sub>3</sub>O<sub>4</sub> Hybrid Composites

The crystallography of the MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid composites was investigated in their X-ray diffraction (XRD) patterns using a Rigaku D/max-2500 (18 kW, Tokyo, Japan) incorporating a θ/θ goniometer equipped with a 40 kV and 30 mA CuKα radiation generator.

The morphological of the MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid composites were examined by transmission electron microscopy (TEM; JEM-2100F, JEOL LTD, Tokyo, Japan). Elemental compositions of as-prepared samples were analyzed by X-ray fluorescence spectrometry (XRF; MiniPal 2, PANanalytical, Almelo, Netherlands). The zeta potentials of the MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid samples and microalgae were measured by dynamic laser-light scattering (DLS; Malvern Zetasizer NanoZS, Malvern Instruments, Malvern, UK).

### 4.5. Microalgae Cultivation

*Chlorella* sp. KR-1 was cultured in nutrient media (constituents: KNO<sub>3</sub>, 3 mM; KH<sub>2</sub>PO<sub>4</sub>, 5.44 mM; NaHPO<sub>4</sub>, 1.83 mM; MgSO<sub>4</sub>•7H<sub>2</sub>O, 0.20 mM; CaCl<sub>2</sub>, 0.12 mM; FeNaEDTA, 0.03 mM; ZnSO<sub>4</sub>•7H<sub>2</sub>O, 0.01 mM; MnCl<sub>2</sub>•4H<sub>2</sub>O, 0.07 mM; CuSO<sub>4</sub>, 0.07 mM; Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>•18H<sub>2</sub>O, 0.01 mM). A Pyrex bubble column reactor (working volume: 6 L) equipped with 12 fluorescent lamps in the front and right/left sides (light intensity: 80 μmol/m<sup>2</sup>/s) was used to cultivate the *Chlorella* sp. KR-1, which were

maintained in a constant-temperature room (30 °C). A 10% (v/v) volume of CO<sub>2</sub> in air at a rate of 0.75 L/min was used to supply the reactor [15,27]. The oil content of *Chlorella* sp. KR-1 has been reported to be around 36.5–41% [28]. The average dry-biomass concentration of *Chlorella* sp. KR-1 in the present growth culture was 1.75 g/L.

*Scenedesmus obliquus* was cultured in another nutrient medium (constituents: Ca(NO<sub>3</sub>)<sub>2</sub>•4H<sub>2</sub>O, 4.23 mM; KH<sub>2</sub>PO<sub>4</sub>, 1.91 mM; MgSO<sub>4</sub>•7H<sub>2</sub>O, 2.48 mM; KCl, 3.35 mM; Fe<sub>2</sub>SO<sub>4</sub>•7H<sub>2</sub>O, 0.07 mM; EDTA•2Na, 0.53 mM; H<sub>3</sub>BO<sub>3</sub>, 0.04 mM; ZnCl<sub>2</sub>, 0.0008 mM; MnCl<sub>2</sub>•4H<sub>2</sub>O, 0.009 mM; (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>•4H<sub>2</sub>O, 0.000015 mM; and CuSO<sub>4</sub>•5H<sub>2</sub>O, 0.0003 mM). The light intensity was about 60 μmol/m<sup>2</sup>/s, and 20% (v/v) CO<sub>2</sub> was fed at rate of 4 L/min [29]. The reactor was maintained in a constant-temperature room (30 °C). The average dry-biomass concentration of *Scenedesmus obliquus* in the growth media was 2.0 g/L. In order to prepare mix microalgae, *Chlorella* sp. KR-1 and *Scenedesmus obliquus* were mixed in a 1:1 ratio by volume.

#### 4.6. Microalgae-Harvesting Procedure

The obtained MgAC-Fe<sub>3</sub>O<sub>4</sub> hybrid composites were dispersed in 1 mL of DI water (loading concentrations: #1: 4.72 g/L, #2: 4.43 g/L, #3: 4.32 g/L, #4: 4.19 g/L, #5: 4.42 g/L) and injected into 12 mL test tubes containing 9 mL of microalgae at a concentration of 1.7–2.0 g/L. The pH of the solutions was adjusted within the range of 2–12. The mixture was slowly mixed by hand (about 1 min) and stirred (800 rpm) for 10 min [15].

UV-Vis spectroscopy (Optizen 2120UV, Mecasys Co., Daejeon, Korea) was used to measure the optical density (OD) of the supernatant of each of the samples (the sampling height was 2/3 of the tube from the bottom). The harvesting efficiency (%) was evaluated by the equation [12]:

$$\text{Efficiency (\%)} = \left[ 1 - \frac{\text{OD}_f}{\text{OD}_i} \right] \times 100$$

where OD<sub>f</sub> and OD<sub>i</sub> were the final and initial ODs of each sample. The experiments were each repeated three times.

The proportions of *Chlorella* sp. KR-1 and *Scenedesmus obliquus* in mixed microalgae were analyzed by a coulter counter (Multisizer<sup>TM</sup> 4, Beckman Coulter, Fullerton, CA, USA) due to the difference of cell sizes. *Chlorella* sp. KR-1 have cells size range of 2–4 μm while *Scenedesmus obliquus* have cells size range of 6–12 μm (Figure S1).

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/1996-1073/11/6/1359/s1>, Figure S1. Number size distribution of mixed microalgae by Beckman Coulter.

**Author Contributions:** Y.-C.L. and Y.-K.O. conceived and designed the experiments; B.K. and W.F. performed the experiments; V.K.H.B. and B.K. analyzed the data; V.K.H.B. and Y.-C.L. principally wrote the paper; S.G.J., Y.-C.L., and Y.-K.O. commented on and improved the manuscript.

**Acknowledgments:** This work was supported by the Advanced Biomass R&D Center (ABC) of the Global Frontier Project funded by the Ministry of Science and ICT, Republic of Korea (ABC-2012M3A6A205388) and also by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (NRF-2017R1D1A1A09000642).

**Conflicts of Interest:** The authors declare no conflict of interest.

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