

# Cr(VI) resistance and bacterial reutilization of Magnetotactic bacteria *Magnetospirillum gryphiswaldense* (MSR-1)

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**Abstract** – Hexavalent chromium removal has been a focus for studies in recent years due to the metal's prevalence and toxicity. Bioreduction using magnetotactic bacteria (MTB) in treating contaminated waters has been gaining interest as their intrinsic magnetic properties allow for easy biomass separation post-treatment, among other benefits of biological methods. The Cr(VI) reduction abilities of MTB strain *Magnetospirillum gryphiswaldense* (MSR-1) has already been proven, but its resistance to the heavy metal and response after multiple treatment cycles have not been studied. The current study aims to describe MSR-1's resistance to Cr(VI) and magnetosome synthesis by testing for its minimum inhibitory concentration and assessing its growth and Cr(VI) reduction upon exposure to consecutive treatment cycles. The results of the experiments show that minimum inhibitory Cr(VI) concentration of MSR-1 was found to be 3 mg/L, although magnetosome synthesis was already retarded at 2 mg Cr(VI)/L. It was also demonstrated that MSR-1 can survive 14 rounds of 12-hour Cr(VI) exposure cycles without significant changes in the total Cr(VI) reduction. However, a notable proliferation of magnetosome-deficient cells was observed in later cycles, suggesting potential limitations for long-term magnetic separability and efficiency.

**Keywords:** magnetotactic bacteria, biomass reuse, chromium resistance, inhibitory concentration

## I. INTRODUCTION

The presence of chromium in the waters poses a serious threat to the humans and aquatic organisms alike due to its toxicity and poor disposal [1, 2]. Chromium is abundant in natural waters as a naturally occurring metal or as a contaminant deposited from industrial processes such as electroplating, mining, metal smelting, batteries, and textiles. In environmental systems, chromium predominantly exists as hexavalent Cr(VI) or trivalent Cr(III). Cr(VI) is noted to be significantly more dangerous - 100 times more toxic and 1000 times more mutagenic than Cr(III) [3, 4]. Reduction of Cr(VI) to the less toxic Cr(III) is a strongly considered strategy to manage chromium contamination in wastewater. Currently, the US Environmental Protection Agency (US EPA) have listed chromium as a priority pollutant, limiting the maximum permissible amount of total Chromium in drinking water to 0.1 mg/L. In the Philippines, the Department of Health also require drinking water to have less than 0.05 mg/L of total Chromium contents [2, 5, 6].

Common methods of controlling chromium pollution include physical and chemical methods such as ion exchange, chemical reduction precipitation, filtration, and solvent extraction. However, these processes usually have high operational cost, limited applicability, and, sometimes, causes secondary pollution. Bioremediation has been considered to be a potential and eco-friendly approach for on-site reduction of Cr(VI) due to advantages such as low cost and high efficiency at lower metal concentrations [2, 7, 8]. Various bacteria have already been identified to be capable of reducing Cr(VI) such as *Pseudomonas*, *Bacillus*, and *Streptomyces* [8]. Biotransformation is one of the reduction mechanisms employed by bacteria to address chromium contamination. Bacteria produce enzymes that aid them in reducing hexavalent chromium to the less toxic trivalent chromium. One of these enzyme groups is chromate reductases which are known to be a pathway that directly converts Cr(VI) to Cr(III). It can be found in the cytoplasm, cell membrane, and even outside the cell [9, 10].

Magnetotactic bacteria (MTB) are Gram-negative bacteria capable of responding to local magnetic fields, an ability called magnetotaxis [11, 12, 13]. Magnetotaxis is believed to be due to the presence of membrane-bound magnetic nanocrystals inside their cells called magnetosomes (MS) [14, 15, 16]. This phenomenon makes MTB potentially separable from the effluent which might also lead to increase in metal recovery. MTB had already been proven to be capable of treating other heavy metals from wastewater such as Cu(II), Au(III), Ni(II), Fe(II), Fe(III), and Cd(II) [17, 18, 19, 20]. *Magnetospirillum gryphiswaldense* (MSR-1) is one of the most extensively studied strains due to its easy cultivation in the laboratory, high magnetosome yield, and high biosorption capacity [21, 22, 23]. Findings from a previous study [24] found that MSR-1 can successfully remove 10 to 40 mg/L Cr(VI) concentrations from synthetic wastewater. However, the minimum inhibitory concentration (MIC) of Cr(VI) to MSR-1 was not tested. MIC is defined as the lowest concentration that prevents net growth of a microorganism [25]. It defines the strain's resistance, which is the ability of the bacteria to grow at high concentrations of the pollutant. Meanwhile, tolerance is recognized as the ability of the bacteria to survive in extreme concentrations much higher than the MIC [25]. MIC serves as the basis for the assessment of the bacteria if they are susceptible or resistant to the introduced agents [26].

Aside from metal recovery, the magnetotactic characteristic of MTB also makes it potentially possible to recover MTB cells for reuse. The usage of MTB in consecutive heavy metal treatment cycles was suggested by Ali et al. [27] in a review paper but it hasn't been attempted in a study until now.

The current study aims to elucidate the Cr(VI) reduction performance of MSR-1 by determining the strain's minimum inhibitory Cr(VI) concentration and measuring MSR-1's growth and Cr(VI) reduction when exposed to consecutive Cr(VI) treatment cycles. The effect of Cr(VI) concentration and consecutive treatment cycles on the magnetosome production of MSR-1 was also observed.

## II. METHODOLOGY

### 2.1 Bacterial culture and strain

*Magnetospirillum gryphiswaldense* (MSR-1) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany. The strain was cultured in flask standard medium (FSM) as described by Heyen and Schuler [28].

### 2.2 MSR-1 resistance experiment and magnetosome measurement

MSR-1 was shaken-cultured in 5 mL FSM in a serum bottle sealed with butyl-rubber stopper at 30°C and 120 rpm for 48 hours under microaerobic conditions (1% O<sub>2</sub>:99% N<sub>2</sub>) [24]. This culture was mixed with 35 mL FSM in 100 mL bottle and shaken-cultured under the same conditions for 16 hours. The resulting culture was washed with 40 mL sterilized ionic washing buffer (IWB) twice after and centrifuged at 4°C and 8500 rpm for 10 minutes. IWB is composed of 0.1 g KH<sub>2</sub>PO<sub>4</sub>, 0.34 g NaNO<sub>3</sub>, and 1 mL EDTA chelated trace mixture solution per liter.

The washed cells were then transferred to serum bottles containing 100 mL FSM with varying amounts of Cr(VI) (0, 1, 2, 3, 5, and 10 mg/L). All cultures were adjusted to the same concentration by setting their initial optical density to 0.05 at wavelength of 565 nm measured using UV-Vis spectrophotometer (Thermo Scientific BioMate 3 Spectrophotometer). The samples were shaken-cultured at 30°C and 120 rpm for 72 hours at aerobic conditions and the growth of the bacteria were monitored by measuring their optical density at 565 nm (OD<sub>565</sub>) at the end of the observation period.

To estimate the magnetosome content of the bacteria after chromium exposure, amended ferrozine assay by Zhao et al. [29] was used to quantify the iron concentrations of the samples.

Each experiment was carried out with at least three independent samples. Quantitative data were subjected to two-tailed t-tests ( $p < 0.05$ ) to compare the means of the sample values to the controls of their respective experiments.

### 2.3 Biomass reuse experiment

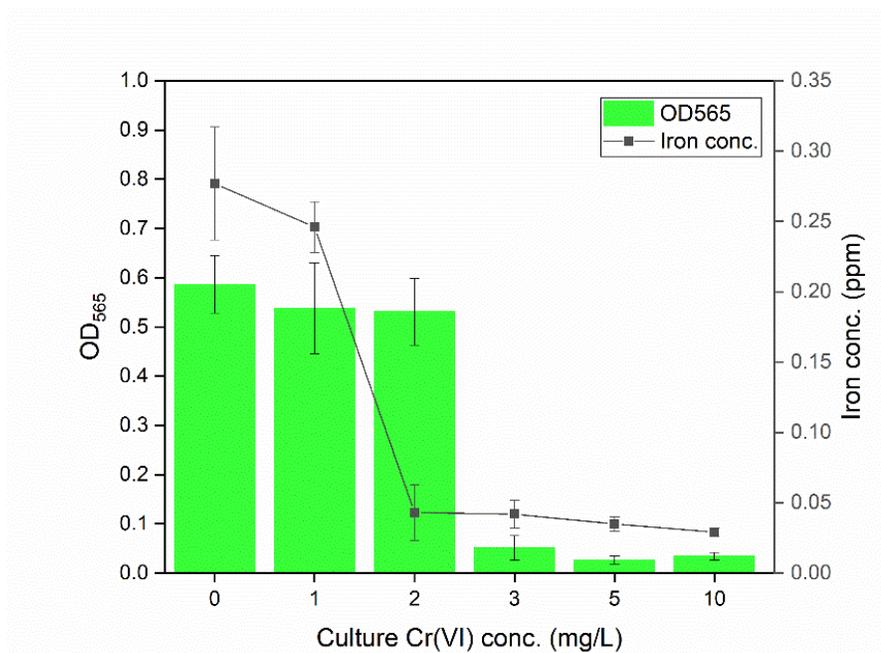
MSR-1 was cultured and washed following the same procedures described in section 2.2. After washing, the bacteria were resuspended in serum bottles containing 40 mL FSM and 10 mg/L Cr(VI) each with the initial concentration adjusted to OD<sub>565</sub> of 0.2. The samples were shaken-cultured at 30°C and 120 rpm for 12 hours under microaerobic conditions. The growth of the bacteria was determined by measuring the OD<sub>565</sub> at the end of the culture period. The Cr(VI) concentrations of the samples at the start and end of the observation period were also measured using the 1,5-diphenylcarbazide (DPC) method, with absorbance values measured at 540 nm [30, 31]. The experiment was done in triplicates.

The cells were then washed with 40 mL IWB twice again after and centrifuged at 4°C and 8500 rpm for 10 minutes before resuspension into 40 mL FSM with 10 mg/L Cr(VI), and the whole process from culture to washing is repeated. The iron content of the samples was also measured at the end of cycles 1, 4, and 14 to track the change in magnetosome content using the amended ferrozine assay.

### III. RESULTS AND DISCUSSION

#### 3.1 MSR-1 resistance experiment and magnetosome measurement

MSR-1 growth was monitored in FSM with varying concentrations of Cr(VI). The optical densities at 565 nm ( $OD_{565}$ ) of these samples and their iron contents at the end of observation period (72 hours) are summarized in Figure 1.



**Figure 1.** Optical density ( $OD_{565}$ ) and iron concentration of the MSR-1 cells at different culture Cr(VI) concentrations. The error bars represent  $\pm 1$  standard deviation.

As shown in Figure 1, a slight decrease in the optical density and iron content of MSR-1 was observed at the lowest Cr(VI) concentration. MSR-1 reached an  $OD_{565}$  of  $0.586 \pm 0.056$  and iron concentration of  $0.277 \pm 0.040$  ppm in cultures without chromium while an  $OD_{565}$  of  $0.538 \pm 0.092$  and  $0.246 \pm 0.018$  ppm iron content was noted in samples with 1 mg Cr(VI)/L. The presence and possible entry of Cr(VI) in the cells may have led to interaction with the intracellular components and affected the metabolism and growth of MSR-1 [32].

The results in Figure 1 also show that the growth of MSR-1 is greatly inhibited at 3 mg Cr(VI)/L. An  $OD_{565}$  of  $0.051 \pm 0.025$  was recorded at this concentration, a sharp decline from  $OD_{565}$  of  $0.531 \pm 0.068$  at 2 mg Cr(VI)/L. A previous study already investigated MSR-1's growth and Cr(VI) reduction, but the culture concentrations tested ranged from 10 to 40 mg/L with initial sample  $OD_{565}$  of 0.2 [24]. Cr(VI) reduction was possible until the highest culture concentration of 40 mg/L. However, MSR-1 growth was also inhibited since  $OD_{565}$  of the samples remained at 0.2 for all the Cr(VI) concentrations studied. The current study tested MSR-1's minimum inhibitory concentration (MIC). As effluents from various sources contain different amounts of Cr(VI), knowing the growth limits of MSR-1 will help maximize its usability. However, it must be noted that MIC defines the strain's resistance but not tolerance

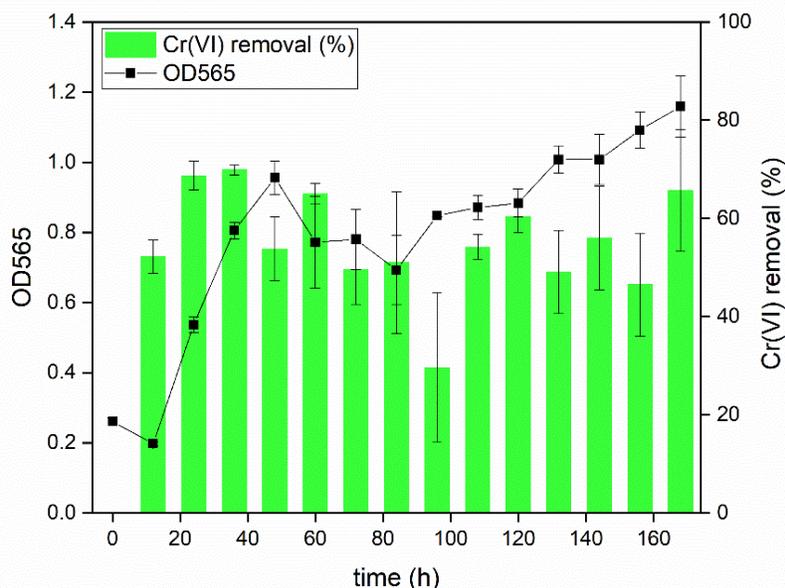
[25]. Resistance is a characteristic of the bacterial strain that is tied to its ability to grow at high concentrations. On the other hand, tolerance is understood as the ability to survive in extreme conditions. In Figure 1, MSR-1 recorded an OD<sub>565</sub> of 0.034 at 10 mg Cr(VI)/L concentrations, which is less than the initial OD<sub>565</sub> of 0.05, showing that the MSR-1 can survive but not grow at this concentration. As the previous study [24] has demonstrated, MSR-1 can still reduce and survive concentrations up to 40 mg Cr(VI)/L.

Interestingly, the magnetosome content significantly dropped to  $0.043 \pm 0.020$  ppm iron concentration in MSR-1 grown in 2 mg Cr(VI)/L, a concentration lower than the MIC. This means that, while MSR-1 still grew at this Cr(VI) culture concentration, these cells probably did not develop magnetosomes. Living organisms, including bacteria, rely on efficient energy allocation to perform various tasks. Some of the key functions for bacteria are growth, division, cell shape regulation, cell maintenance, and energy storage [33]. The transport of the reductase enzyme and proteins produced by bacteria to be used for extracellular reduction of Cr(VI) is an energy-intensive process [34]. Previous studies also suggest that magnetosome synthesis is an energy-dependent process as the ATPase, an enzyme involved in the production of ATP or the cellular energy of the cell, was found to be involved in the iron uptake of MTB as well [21, 35]. These processes probably compete for the energy available in the cells. The growth of MSR-1 without magnetosomes in 2 mg Cr(VI)/L may be explained by the prioritization of other functions over synthesis of magnetosome for energy allocation of the cells.

Previous papers focused on magnetotactic bacteria's ability to bioremediate Cr(VI) and survive high levels of the heavy metal [24, 36]. This is the first study that clearly defines the resistance levels of a magnetotactic bacterial strain to Cr(VI) by measuring its MIC.

### 3.2 Biomass reuse

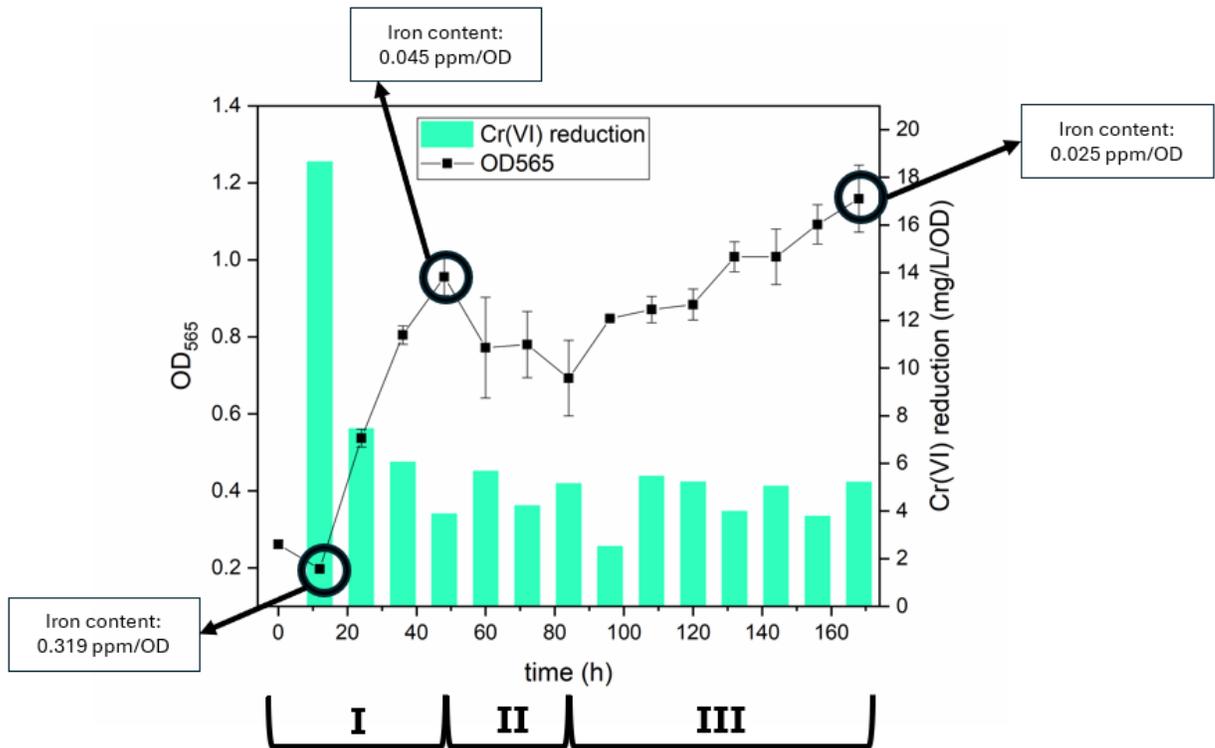
Magnetotaxis, the ability of magnetotactic bacteria to follow local magnetic field lines, potentially makes it possible for the biomass to be separated from the heavy metals after treatment [27]. This opens the idea of the biomass to be reused without the cost- or energy-intensive processes. This experiment investigates on MSR-1's growth and chromium reduction capacity after being exposed to a sequence of Cr(VI) treatment. The summary of Cr(VI) removal per run is shown in Figure 2.



**Figure 2.** OD<sub>565</sub> and Cr(VI) removal (%) of the MSR-1 cultures after sequential Cr(VI) treatments. The error bars represent  $\pm 1$  standard deviation.

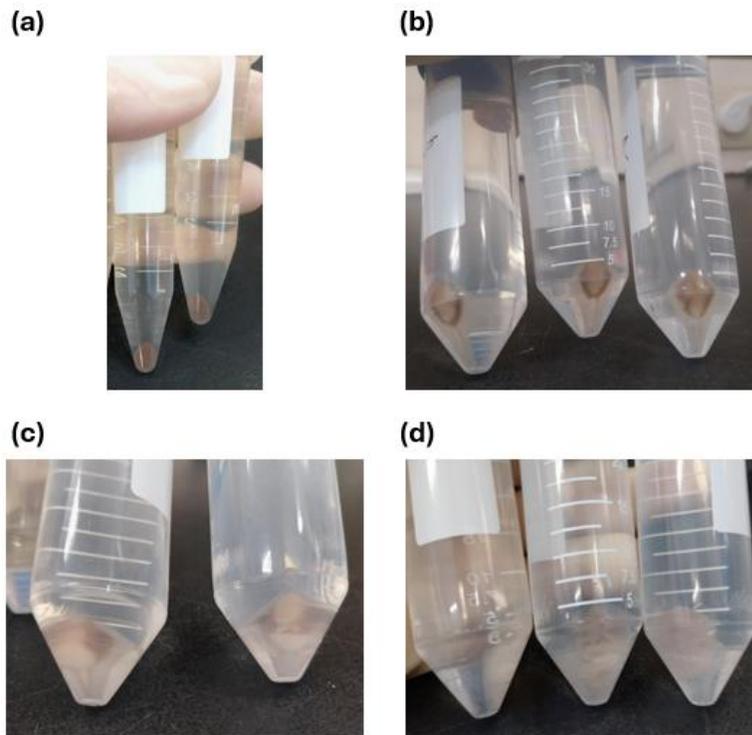
Figure 2 shows that MSR-1 was able to survive and grow for 14 runs of 12-hour experiments while still being able to reduce Cr(VI) when exposed to 10 mg/L Cr(VI) concentrations. A previous study showed that MSR-1 can remove 10 mg Cr(VI)/L at around 40 hours, with the capacity of reducing half of it in the first 12 hours [24]. The 10 mg Cr(VI)/L culture concentration also led to inhibition of cell growth and no significant changes in MSR-1 OD<sub>565</sub> were recorded during the 40-hour experiment period. This culture concentration was selected due to it being an inhibiting concentration, with the expectation that the cell densities or the Cr(VI) reduction rate will reach its definitive limit after a few runs. Surprisingly, MSR-1 cell density increased from  $0.197 \pm 0.005$  at the end of 1<sup>st</sup> run to  $0.537 \pm 0.023$  in the 2<sup>nd</sup> run to a first peak of  $0.956 \pm 0.048$  at the 4<sup>th</sup> run in the current study. An increase in total chromium reduction was also observed with the recorded Cr(VI) removal being  $52.18\% \pm 3.45\%$ ,  $68.66\% \pm 2.92\%$ , and  $69.85\% \pm 1.13\%$  for the first three runs. The Cr(VI) removal for the rest of the experiment remained around 50%, although some significant fluctuations were observed. The OD<sub>565</sub> of MSR-1 had a substantial increase from the 1<sup>st</sup> to the 4<sup>th</sup> run, gradual decrease from the 5<sup>th</sup> to the 7<sup>th</sup>, and continuous increase for the remainder of the experiment.

It was demonstrated before that magnetosome synthesis affects Cr(VI) reduction of MSR-1 by enhancing chromate reductase activity. However, it also results in the suppression of growth of cells leading to a decrease in the overall Cr(VI) reduction rate compared to magnetosome-deficient strains [24]. Comparisons on the Cr(VI) reduction of MSR-1 with and without magnetosomes were not explored on the said research. To provide a better explanation of MSR-1's behavior during this study, the Cr(VI) removal was converted into the cellular Cr(VI) reduction and iron contents of the samples after cycles 1, 4, and 14 were also recorded. These changes are reflected in Figure 3.



**Figure 3.** Optical density (OD<sub>565</sub>) and chromium reduction capacity of the MSR-1 cells after sequential Cr(VI) treatments. The iron contents of the samples (in ppm/OD) at different periods (encircled) in the experiments were indicated in the boxes. The error bars represent  $\pm 1$  standard deviation.

Figure 3 show that, although an increase in Cr(VI) reduction was noted in the 2<sup>nd</sup> run, converting the units to Cr(VI) reduction per cell OD showed that this run actually resulted in a sharp decrease in Cr(VI) reduction abilities of the cell from 18.656 mg/L/OD to 7.464 mg/L/OD. The Cr(VI) reduction rate of the cells remained around 5 mg/L/OD from the 3<sup>rd</sup> to the 14<sup>th</sup> runs.



**Figure 4.** MSR-1 centrifugation pellets (a) at the start of the experiment, (b) after the 1<sup>st</sup> run, (c) after the 4<sup>th</sup> cycle, and (d) at the end of the experiment.

Based on the results in Figure 3, the response of MSR-1 to repeated exposure to Cr(VI) may be divided into three stages. And, taking the magnetosome concentrations at different periods in the experiment into consideration, the division of MSR-1's response into three stages may be due to shifting priorities in its energy allocation discussed in section 3.1. Although there are several key physiological functions of cells, this section assumes that the changes in energy distribution of MSR-1 cells are simplified into only three allocations: cell growth, Cr(VI) reduction, and magnetosome synthesis. The pellets of culture of magnetic MSR-1 cells appear in dark color after centrifugation at the start of the experiment (Figure 4a) which is expected from the magnetosome-rich MSR-1 cells. After the 1<sup>st</sup> run, the iron content of the samples was found to be 0.319 ppm/OD. The first stage (I) involved a rapid increase in cell density and decrease in terms of Cr(VI) reduction capabilities of the bacterial cells. The amount of magnetosomes seems to be mostly retained (black area), although the density of magnetosome decreased as seen by the appearance of white non-magnetic cells in Figure 4b and verified by the iron content in ferrozine assay at 0.045 ppm/OD. This stage may signify that the strain prioritized cell growth and tried to maintain the magnetosome synthesis of the cells during its first few Cr(VI) exposures. Run 5 saw an increase in both cellular and total Cr(VI) although the cell concentration dropped. During the second stage (II), the strain's growth stagnated while maintaining a certain level of Cr(VI) reduction abilities of the bacteria. This may reflect a shift in survival strategy, prioritizing the maintenance of existing Cr(VI) reduction capacity over rapid proliferation. However, this energy reallocation was still

insufficient to reverse the ongoing depletion of magnetosomes. Run 8 recorded a decrease in both cellular and total Cr(VI) despite the noticeable growth in OD<sub>565</sub>. This behavior was opposite the observed response of MSR-1 on run 5. These transition runs may possibly function as signals of MSR-1 as it shifts its energy allocations. These changes might be due to operation of different regulators within MSR-1. Crp, a global regulator, demonstrated the relation between growth, energy metabolism, and magnetosome synthesis in MSR-1 in a previous study [37]. Although Crp is mainly concerned in controlling energy and carbon metabolism in most microorganisms, it was found to be involved in the regulation of magnetosome island genes in MSR-1. Its disruption may have contributed to the low growth and decreasing magnetosomes amidst the increase in Cr(VI) reduction in the 5<sup>th</sup> run. The third stage (III) saw a continuous rise in the OD<sub>565</sub> of MSR-1, and similar levels of Cr(VI) reduction to the previous stage. Cell growth became the strain's main priority again in its last stages, with Cr(VI) reduction still maintained. The pellets turned into lighter gray color, signaling low magnetosome density in the samples as shown in Figure 4d. It was verified by the iron content values at the end of the experiment period at 0.025 ppm/OD, a 44.4% decrease from the concentration recorded at the end of stage I. At the end of the experiment period, it was assumed that the number of magnetosome-deficient cells continues to increase leading to further rise in the culture optical densities until it reaches the energy and nutrient supply limit of the culture.

Regeneration/reuse of biosorbents are important for both resource recovery and continuous biomass supply [38]. Adsorption/desorption experiments of biosorbents were conducted before to test the reusability of bacterial cells in heavy metal treatment. The biomass reuse of *Pseudomonas* sp., *S. xylosum*, and *B. trispora* for Mn(II) recovery was investigated by another study [39]. The desorption rate of all strains remained high, but the adsorption capacities of the cells greatly decreased within 4 cycles. Chr B-expressing *E. coli* cells were discovered to be more capable of maintaining their adsorption capacity after 4 rounds of adsorption-desorption cycles [38]. The current study focused on the reduction capabilities of MSR-1 for its evaluation of being reusable. Although majority of the remaining cells were magnetosome-deficient, the Cr(VI) reduction rate did not suffer as seen in Figure 2. A 65.67% Cr(VI) reduction was still recorded during the 14<sup>th</sup> run, higher than the 1<sup>st</sup> run's reduction of 52.18%.

One of the unique properties of MSR-1 is its magnetotaxis that can possibly make it separable from the effluent and allow the bacterial cells to be reused [27]. Recovery of the heavy metal by separation of the biomass from the effluent was already tested before [40]. This study is the first to attempt the reuse of the magnetotactic biomass after metal bioremediation, albeit without magnetic separation.

#### IV. CONCLUSIONS AND RECOMMENDATIONS

The growth, magnetosome synthesis, and performance of *Magnetospirillum gryphiswaldense* (MSR-1) in Cr(VI) reduction were explored in the study. Minimum inhibitory concentration (MIC) experiments found that Cr(VI) concentrations as low 3 mg/L limit the growth of the strain while magnetosome synthesis begins to decline at a lower concentration

of 2 mg Cr(VI)/L. Tolerance studies of MSR-1 to Cr(VI) and determining the maximum concentrations for Cr(VI) reduction are still open for exploration for future studies. Meanwhile, biomass reuse experiments demonstrated that MSR-1 can survive at least 14 rounds of 12-hour Cr(VI) exposure cycles. There are no noticeable changes in the total Cr(VI) reduction, although the Cr(VI) reduction rate per cell has sharply declined starting at the 2nd run. The magnetosome density also decreased at the end of the experiment, albeit the observed cell density increase. This indicates the growth of magnetosome-deficient MSR-1 cells. Shifting energy allocations were suggested to explain the behavior of MSR-1 for the duration of the experiment. To counteract the proliferation of non-magnetic cells, magnetic separation could be strategically applied between cycles. This would selectively enrich the culture with magnetosome-containing cells for the subsequent treatment round, thereby maintaining the efficiency of the magnetic recovery process. Further research is warranted to optimize culture conditions — such as intermittent iron supplementation — to determine if the loss of magnetosomes during long-term reuse can be mitigated.

## V. ACKNOWLEDGEMENTS

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