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Activity Of Different Metals In Disinfection Of Activated Sludge By A Fenton-Like Process

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Treatment technologies of the activated sludge (AS) for its use as a fertilizer should include a hygienization process to meet legislative criteria. Numerous approaches have a strong effect on sludge stabilization and dewatering, while disinfection efficiency is unsatisfactory. In this respect, a combination of different physical (e.g., thermal) and chemical processes could develop hybrid methods for converting the sludge into a qualitative fertilizer with safe microbiological characteristics. Our study was focused on the comparison of the efficiency of Fe(II), Cu(II), Co(II), Mn(II), and Zn(II), which mediated the Fenton-like reaction in combination with H_2O_2, - for disinfection of AS from the municipal wastewater treatment plant. The enzymatic activity, i.e., fluorescein diacetate (FDA) hydrolysis, served as an integral criterion of cell inhibition. After 72h treatment of AS with 7 mM Me and 200 mM H_2O_2, an inhibition efficiency was ranged as follows:
[Fe+Co]>>[Co]≥[Fe+Cu+Co+Mn+Zn]>[Mn]>[Fe+Cu+Co]>[Cu]>[Zn]>[Fe+Zn]>[Fe].The effect of Fe(II) and Co(II) as single metals and binary mixtures on AS ecotoxicity was evaluated. No toxic effect was detected in a 24h immobility test with Artemia sp. and 72h root elongation of Raphanus sativus seeds. The AS treatment with Fe(II)+Co(II) in the presence of H_2O_2 leads to additional antimicrobial resistance of AS microbial community towards amikacin, piperacillin / tazobactam constant 4, doripenem, impenem, meropenem, and cefepime, as compared to untreated AS.

Keywords— Copper, dehydrogenase, fluorescein diacetate hydrolysis, iron, phytotoxicity.

I. INTRODUCTION

Wastewater treatment sludge refers to waste generated from wastewater treatment plants (WWTP) where the activated sludge (AS) is a by-product of the treatment process. It is characterised by the source of the wastewater, it can originate from various sources, i.e., domestic or industrial [1]. Currently, the AS from wastewater treatment plants are disposed of in sewers or to landfills, which is costly and non-environmentally friendly, therefore it's crucial to develop alternative management strategies such as recycling, reuse and resource recovery [2]. The EU sewage sludge directive states that treated WWTS can be used in agriculture, showing that it is useful as a fertilizer or soil improver. Some EU countries allow untreated sludge to be used as fertilizer, with some exceptions, however, it could pose serious environmental and public health concerns [3].

Currently, the number of methods for AS treatment is limited and not up to par with changing regulations. However, over the years, the number of research papers pointing out the necessity for a better wastewater treatment system for AS in wastewater treatment plants has grown drastically [4]. Especially now when the spread of antibiotic resistance gene (ARG) has been linked with WWTP [5].

Traditionally methods of AS disinfection are physical, biological and chemical [6]. Resent progress in Fenton processes has highlighted its potential use in sludge disinfection and ARG removal. Fenton reaction achieves the degradation of organic contaminants generating highly reactive hydroxyl radicals (•OH) through the reaction of strong oxidants like hydrogen peroxide with a metal catalyst [7], [8].

Fenton reaction can be utilized industrially as pre- and\ or post-treatments. Most widely, the method is linked to WWTP and multiple types of industry effluents, such as the dairy, textile, chemical, food and beverage industries [9],[10]. Considering that the Fenton reaction is one of the most popular advanced oxidation processes (AOPs), and it has some outstanding benefits, it also has some disadvantages [11]. A major challenge is the low pH for efficient Fenton reaction [12]. Fenton reaction's quality and effectiveness can also be influenced by the reaction temperature, AS density, and dosages of the catalyst and H_2O_2 [13].

Fenton reaction is commonly compared to Fenton-like reaction [14]. Key differences between these reactions are the classic Fenton reaction specifically uses ferrous ions, while Fenton-like reactions can involve various metal ions and the efficiency of generating hydroxyl radicals can vary depending on the metal ion and reaction conditions. Yet, Fenton-like reactions may be conducted under broader conditions (e.g., different pH) compared to the traditional Fenton reaction [15]-[17].

This study was aimed at evaluating the effect of different transition metals, i.e., Fe(II), Co(II), Cu(II), and Zn(II) in the presence of H_2O_2 at near-neutral pH, - on microbial activity, antimicrobial resistance, and ecotoxicity of AS after the 72h treatment. Microbial activity was measured by fluorescein diacetate hydrolysis and dehydrogenase, antimicrobial susceptibility changes were determined by Sensititre® GN4F. plates, while ecotoxicity was tested by immobility of crustaceans Artemia sp. and seed germination of radish Raphanus sativus.

II. MATERIALS AND METHODS

AS was obtained from the municipal WWTP Sloka. The AS had the following characteristics: pH 5,7; organic dry matter -79,3%, sludge dry matter 0.99%; total phosphorus- 26,8 g/kg and total nitrogen- 66 g/kg. Metal stock solutions (700 mM) were prepared using Fe(II), Cu(II), Co(II), Mn(II), and Zn(II) sulphates, all of analytical grade.

A. Fenton and Fenton- like metal catalysts comparison

AS was transferred to a 12-well microplate (3 mL). Each plate had the final metal concentration of 7 mM, irrespectively of metal combination (30 µl of metal catalyst) and 200 mM H2O2 (60 µl of 35% H2O2). Additionally, plates with the same treatments were preincubated at 70 □C for 60 min. Plates were incubated in the dark at 23 □ for 72 h, in triplicate.

B. Enzyme activity

Fluorescein diacetate (FDA) hydrolysis. AS samples (100 µl) from each well were transferred to 48-well microplate. 100 µl of FDA reaction mixture (4 mg FDA, 2 ml acetone, 48 ml of 60 mM phosphate buffer solution, pH 7.6). Reaction mixtures were incubated at 23 □C for 60 min, afterwards 50 □L were transferred to a 96-well plate. Optical density (OD) was measured using the microplate reader (TECAN infinity F50) at OD492 nm.

Dehydrogenase (DHA) activity. AS samples (100 µl) from each well were transferred to a 96-well plate. 100 µl of DHA reaction mixture (1.97g Trizma, 0.04 g INT and 25 mg glucose, 20 ml deionized water) was added to each well. Reaction mixtures were incubated at 23 □C for 60 min, afterwards AS was observed under light microscopy. Five micrographs from each replicate (total 15 micrographs per each treatment type) were made. Micrographs were treated using https://labs.tineye.com/color/, for color extraction, i.e., the yield of red color in floc expressed in percents.

C. Antimicrobial Susceptibility Testing

To evaluate the antimicrobial susceptibility of AS microbial community, the Sensititre™ Gram Negative GN4F Plates (Thermo Fisher Scientific, USA) were used. This semi-quantitative test is intended for testing of clinical non-fastidious Gram-negative isolates. The method was modified, particularly, i) inoculation of microbial community instead of a single isolate, ii) the use of diluted broth; iii) the lower temperature of incubation. In this study, the plate with 24 antimicrobial agents (either single or compositions) was applied for incubation of AS after 72h treatment. The plate was inoculated with AS suspension, i.e., 10 □L of AS and 40 □L of 10x diluted Tryptic Soy Broth (Bio-Rad Laboratories, USA), in total 50 □L per well, and incubated at 23 □C for 24h. The results were read manually, i.e., the wells with microbial growth were considered positive.

D. Ecotoxicological evaluation

The ecotoxicity of AS after the 72h treatment has been evaluated using the germination test with seeds of radish and the immobility test with crustaceans Artemia sp.

Ten seeds of radish Raphanus sativus were placed in separate wells in a 6-well microplate, each well contained one mL AS covered by filter paper. The control wells contained 1 mL of deionized water. The seeds were incubated in dark at 23 □C, for 3 days. Afterwards, the number of germinated seeds was counted, and the length of roots and shoots was measured.

The immobility test with Artemia sp. eggs (Artemio® PUR, JBL, Germany) was performed. Nauplii were hatched after incubation in 2.5% NaCl for 24h at 25 □C under continuous illumination. An immobility test was made in a 24-well microplate. Each well contained 150 □L AS and 50 □L 10% NaCl to provide 2.5 % NaCl in a sample. The control wells contained 150 □L deionized water instead of AS. Ten nauplii were placed in each well. Plates were incubated in the dark at 23 □C. The mobile shrimps were counted after 24h incubation, using a dissecting microscope CETI (UK).

E. Statistical analysis

Data processing and statistical analysis were performed using Microsoft Excel, and a statistical significance test (single factor ANOVA analysis, $p<0.05$).

III.RESULTS AND DISCUSSION

A. FDA hydrolysis activity in AS

Based on previous experimental data (data not shown) the minimum concentration for Fe(II) and hydrogen peroxide was determined at 7 mM and 200 mM, respectively. These conditions were chosen and maintained across all experiments to ensure the higher efficiency of other tested metals could be detectable.

We investigated the efficiency of various metal catalysts in combination with hydrogen peroxide, to inhibit microbial FDA hydrolysis activity. Table I summarizes the FDA activity of AS exposed to H2O2 and single/multi metal combinations (Fe(II), Co(II), Cu(II), Mn(II), and Zn(II). FDA hydrolysis assay determines protease, lipase, and esterase enzymes, which are involved in the hydrolysis of FDA. The FDA hydrolysis has mainly been used in soil, water, sludge studies, for measurement of microbial activity [18]-[21]. Therefore, a decrease of FDA hydrolysis activity indicates the higher disinfection efficacy.

Among the single-metal catalysts, Co(II) exhibited the lowest enzyme activity, i.e., the values were lower by 54.00 % comparing with control. This indicates that Co(II) had the best disinfection capability under tested conditions. While Fe(II) is a well-established Fenton reagent, in this particular setup, Co(II) outperformed it in terms of disinfection, despite Fe(II)'s higher catalytic activity in decomposing hydrogen peroxide [22].

The combination of Fe(II) with Cu(II) did not show a notable inhibition effect, comparing with control (7.53%), possibly due to competition for hydrogen peroxide or interference in hydroxyl radical production Similar effects have been reported in other studies, where combining certain metals led to reduced catalytic efficiency [23].

Conversely, the combination of Co(II) with Fe(II) in the presence of H2O2 resulted in even lower enzyme activity than Co(II) alone. Particularly, an inhibition effect in that case achieved 76.51% comparing with control. This suggests a potential synergistic effect between Co(II) and Fe(II).

Fe(II) and Co(II) have been studied earlier for developing a low-cost and highly efficient adsorbent and Fenton catalyst. Thus, the Co-Fe-biochar exhibited a high adsorption capacity and strong advanced oxidation [24]. Another emerging advanced oxidation technology with Co(II) is the Co/peroxymonosulfate system [25], [26] . Yan et al. (2021) reported about a MoS2 cocatalytic heterogeneous Fenton (CoFe2O4/MoS2) system capable of degrading organic pollutants [27]. Recently a new Fenton-like catalyst derived from tungsten waste and cobalt, was tested [28].

A short-term thermal pre-treatment of AS in the presence of H2O2 and metals resulted mostly in a decrease in FDA hydrolysis activity compared with respective sets without thermal pre-treatment. Only two exceptions were detected, i.e., the thermal pre-treatment resulted in a slight increase of enzyme activity in the sets with Cu(II) and Fe(II)+Co(II). Thermal pre-treatment inhibited the control set (non-amended AS) by 34.48%. Not all combinations of metals resulted in a further decrease of the FDA hydrolysis activity compared with the control. The sets with H2O2 only, as well as single metals Fe(II) and Cu(II) in the presence of H2O2 demonstrated a higher activity than in control (Table I).

Table I. The activity of FDA hydrolysis in AS (WWTP "Sloka") after 72h treatment with 200 mM H2O2 and 7 mM Me(II) (the final concentration for both single and multiple metal mixtures). Thermal pre-treatment was performed at 70 □C for 60 min. All sets with metals were amended with 200 mM H2O2. Values of the FDA hydrolysis activity are expressed in relative units.

Treatment type	Without pre-treatment		Thermal pre-treatment	
	AVE	STDEV	AVE	STDEV
Control	0.388	0.102	0.255	0.038
H_2O_2	0.294	0.081	0.279	0.045
Fe	0.318	0.152	0.268	0.046
Cu	0.251	0.049	0.274	0.080
Co	0.179	0.022	0.150	0.021
Mn	0.222	0.045	0.187	0.021
Zn	0.263	0.093	0.175	0.042
Fe+Cu	0.359	0.100	0.118	0.012
Fe+Co	0.091	0.024	0.137	0.045
Fe+Mn	0.246	0.089	0.160	0.036
Fe+Zn	0.273	0.053	0.197	0.026
Fe+Cu+Co+Mn+Zn	0.180	0.052	0.208	0.022

B. DHA enzyme activity in AS flocs

A strong inhibitory effect of Co(II) on the FDA hydrolysis activity has been revealed (Table I). In this respect, other characteristics of microbial activity in sludge would support this finding, comparing a physiological response of microorganisms towards Fe(II), Co(II), and Fe(II)+Co(II) in the presence of H2O2. Thus, DHA activity in AS can help to evaluate any inhibitory or stimulating effects posed on microbial community activity [29], [30]. As shown in Fig. 1, no significant ($p>0.05$) differences in DHA activity between control, treatment by H2O2, as well as Fe(II)+ H2O2 were detected. In turn, treatment by Co(II) and Co(II)+Fe(II) in the presence of H2O2 resulted in significant inhibition of DHA in sludge flocs. A two-fold decrease of Co(II)+Fe(II) concentrations resulted in a considerably higher DHA activity. Nevertheless, these values were still significantly lower, comparing with control, H2O2 alone, and Co(II) with H2O2 (Fig.1).

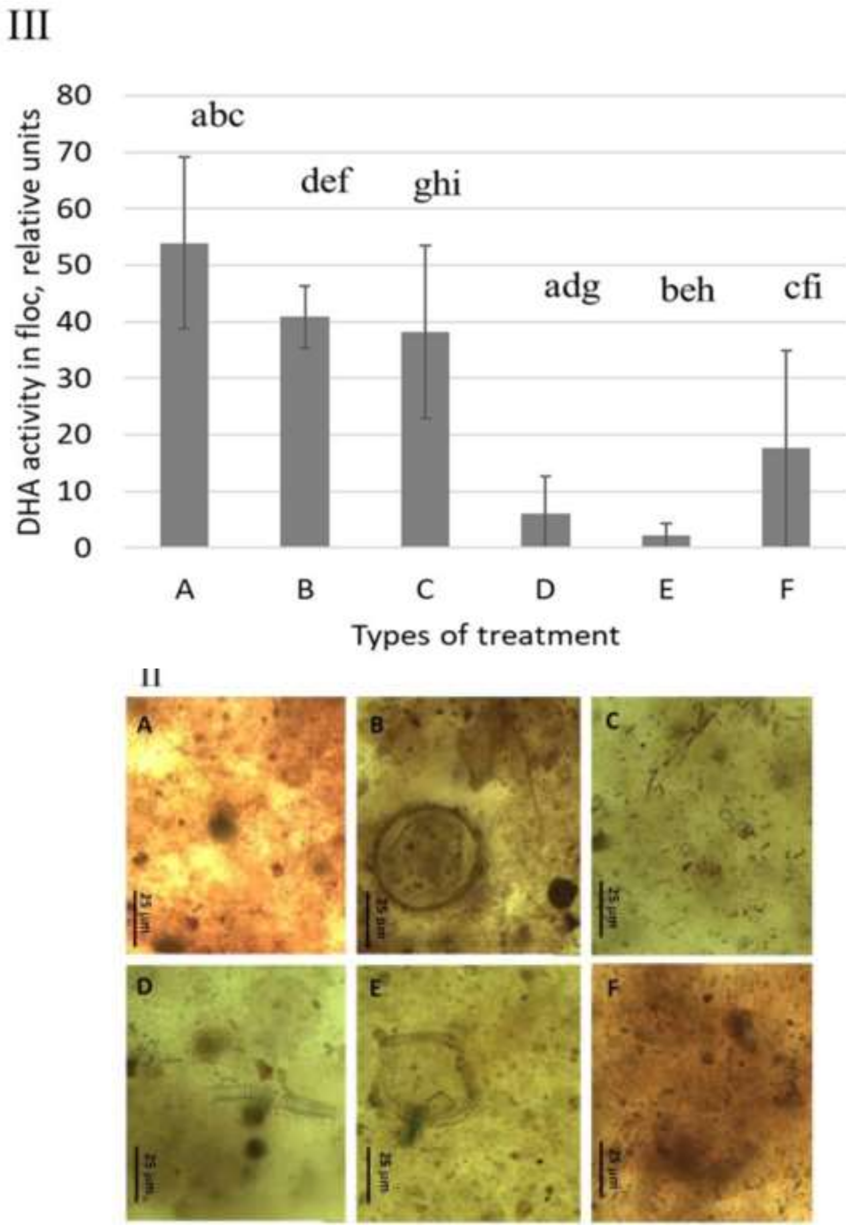

Fig.1. Dehydrogenase activity in AS flocs after 24h treatment. I – microplate before and after DHA assay; II –micrographs after DHA assay, bar 25 μm; III – yield of red color in flocs made by a color extraction software. A – non-amended control; B - 200 mM H2O2;

C – 7.0 mM Fe(II); D – 7.0 mM Co(II); E – 3.5 mM Fe(II)+3.5 mM Co(II); F – 1.8 mM Fe(II)+1.8 mM Co(II). All sets with metals were amended with 200 mM H2O2. Statistically significant ($p<0.05$) differences are indicated by the same letter above the respective bar.

The effect of Fenton and Fenton-like processes on sludge DHA activity can differ depending on the type of sludge, treatment conditions, chemicals used, and other factors. Thus, Huang et al. reported an increased DHA activity after sludge conditioning by Mn(II)-catalytic ozonation [31]. Another study showed a positive effect of Fe(II) on microbial DHA in the sludge [32]. In turn, Zhao et al. reported the inhibition of microbial DHA activity in sludge, where combining biodegradation and Fenton oxidation was applied for the biodegradation of polymer quaternary ammonium salt [33].

C. Susceptibility of microbial community to antimicrobial agents

The changes in antimicrobial resistance of the AS microbial community could be another important criterion for evaluation of a sludge treatment feasibility and risk assessment. The control (non-amended) set demonstrated a susceptibility towards nine antimicrobial agents among 24 agents represented in Sensititre□ plate GN4F. In the set with AS treatment by Fe(II) and H2O2, additional resistance to amikacin was detected, while the resistance to ciprofloxacin was reduced, comparing with the control. In the set with Co(II) treatment, resistance to ciprofloxacin and nitrofurantoin has been reduced. In turn, a higher number of tested antimicrobials, which were resisted by AS microbial community, has been observed in the set with Fe(II)+Co(II) and H2O2. Particularly, additional resistance to amikacin, piperacillin / tazobactam constant 4, doripenem, impenem, meropenem, and cefepime was detected.

Urban WWTPs represent the source of antibiotics, which may promote the selection of antibiotic resistance genes and antibiotic resistant bacteria and release to environment [34]. Antibiotic resistance of microorganisms in sludge can considerably change upon treatment. Venâncio et al. recently reported the effect of combination of coagulation/flocculation and Fenton processes on an efficiency of disinfection and abundance of antibiotic resistance. The authors emphasized the presence of enterobacteria resistant to amoxicillin or sulfamethoxazole [35]. Our testing did not detect the resistance to sulfamethoxazole in all tested samples, while amoxicillin is not included in Sensititre□ plate GN4F. Further study is needed to evaluate the effect of binary mixtures of transient metals applied in Fenton-like processes, on the changes in antimicrobial susceptibility in AS.

D. Ecotoxicological evaluation of treated AS

Ecotoxicity testing of AS samples after 72h treatment was conducted using Artemia sp. No changes in mobility of Artemia sp. between treated and untreated sets were detected.

Ecotoxicological evaluation of treated AS involves assessing its impact on the environment, particularly on living organisms. This evaluation is crucial to ensure that treated sludge used in land applications, such as agriculture, landscaping does not negatively affect soil, plants, water systems, or wildlife [36]. Exposing seeds like radish Raphanus sativus to treated sludge was used to assess whether the chemicals in the sludge inhibit or promote germination.

Treatment A (non-amended control) resulted in the highest root length (~65 mm), followed by treatment E (3.5 mM Fe(II) + 3.5 mM Co(II)) with a root length of ~60 mm. Conversely, treatment F (1.8 mM Fe(II) + 1.8 mM Co(II)) showed the lowest root length (~10 mm), indicating a significant inhibitory effect on root growth (Fig.2).

The highest root growth in the untreated sample could indicate that the AS contained sufficient nutrients or conditions for optimal growth, without the need for amendments [37]. The combination of iron and cobalt (treatment E (Fe(II) + Co(II))) may have provided an additional support of micronutrients, boosting root elongation. Nevertheless, additional studies are necessary.

Compared to the water control, where root growth was ~20 mm with A (non-amended control), where root growth was ~65 mm, resulted in significantly greater root elongation, indicating that the AS supplied the seeds with nutrients.

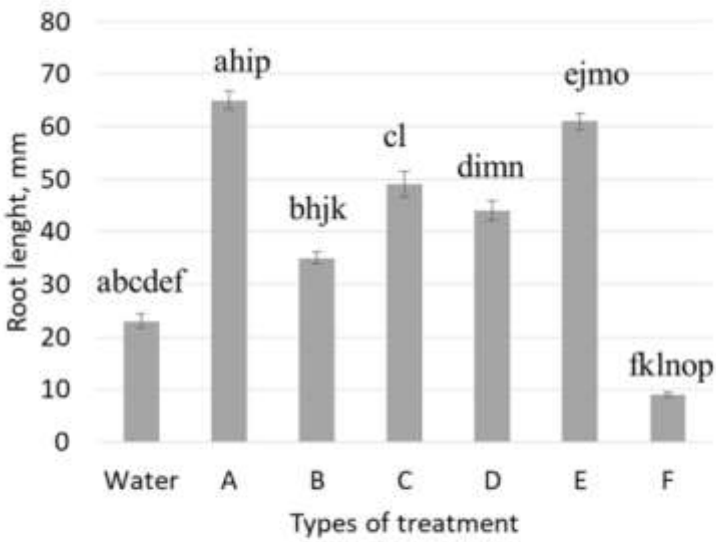

Fig.2. Root elongation of Raphanus sativus after 72h incubation. Description of AS treatment types as in Fig.1. Statistically significant ($p<0.05$) differences are indicated by the same letter above the respective bar. Water; A – non-amended control; B - 200 mM H2O2; C– 7.0 mM Fe(II); D – 7.0 mM Co(II); E – 3.5 mM Fe(II)+3.5 mM Co(II); F – 1.8 mM Fe(II)+1.8 mM Co(II).

Gikas has reviewed the effect of Co in AS. Cobalt belongs to the so called “essential” metals. It is an important co-factor in vitamin B12-dependent enzymes and other enzymes [38]. The theoretically optimum Co concentration for unrestricted performance of AS systems has been estimated in the range from 0.02-0.05 mg/L [39]. Cobalt is a potent intoxicator to AS at concentrations above 20 mg/L. However, the published data on Co toxicity are often contradictive, even for similar type of microbial systems [38]. In our study, Co was loaded to AS at 412.3 mg/L (7 mM) and 206.2 mg/L (3.5 mM). Thus, an inhibition of microbial activity in AS even without H2O2 was expected. Further studies on Co(II) activity in a Fenton-like process, both as a single catalyst and in combination with Fe(II), should bridge the knowledge gap in increasing AS disinfection efficiency and decreasing environmental risks.

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