Anaerobic Degradation of Environmentally Hazardous Aquatic Plant *Pistia stratiotes* and Soluble Cu(II) Detoxification by Methanogenic Granular Microbial Preparation

Olesia Havryliuk [1], Vira Hovorukha [1], Oleksandr Savitsky [2], Volodymyr Trilis [2], Antonina Kalinichenko [3,*], Agnieszka Dołhańczuk-Śródka [3], Daniel Janecki [3] and Oleksandr Tashyrev [1]

1 Department of Extremophilic Microorganisms Biology, Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, 03143 Kyiv, Ukraine; gav_olesya@ukr.net (O.H.); vira-govorukha@ukr.net (V.H.); tach2007@ukr.net (O.T.)

2 Department of Ichthyology and Hydrobiology of River Systems, Institute of Hydrobiology, National Academy of Sciences of Ukraine, 12 Prosp. Geroiv Stalingradu, 04210 Kyiv, Ukraine; a_savitsky@ukr.net (O.S.); trylis@rambler.ru (V.T.)

3 Institute of Environmental Engineering and Biotechnology, University of Opole, 45-040 Opole, Poland; agna@uni.opole.pl (A.D.-Ś.); zecjan@uni.opole.pl (D.J.)

* Correspondence: akalinichenko@uni.opole.pl; Tel.: +48-787-321-587

Abstract: The aquatic plant *Pistia stratiotes* L. is environmentally hazardous and requires effective methods for its utilization. The harmfulness of these plants is determined by their excessive growth in water bodies and degradation of local aquatic ecosystems. Mechanical removal of these plants is widespread but requires fairly resource-intensive technology. However, these aquatic plants are polymer-containing substrates and have a great potential for conversion into bioenergy. The aim of the work was to determine the main patterns of *Pistia stratiotes* L. degradation via granular microbial preparation (GMP) to obtain biomethane gas while simultaneously detoxifying toxic copper compounds. The composition of the gas phase was determined via gas chromatography. The pH and redox potential parameters were determined potentiometrically, and Cu(II) concentration photocolorimetrically. Applying the preparation, high efficiency of biomethane fermentation of aquatic plants and Cu(II) detoxification were achieved. Biomethane yield reached 68.0 ± 11.1 L/kg VS of *Pistia stratiotes* L. biomass. The plants' weight was decreased by 9 times. The Cu(II) was completely removed after 3 and 10 days of fermentation from initial concentrations of 100 ppm and 200 ppm, respectively. The result confirms the possibility of using the GMP to obtain biomethane from environmentally hazardous substrates and detoxify copper-contaminated fluids.

Keywords: biomethane; *Pistia stratiotes* L. plants; copper bioremoval; anaerobic degradation of hazardous plants; environmental biotechnology; bioremediation; biomethane production

1. Introduction

Today, our planet suffers from a number of environmental problems that require globally effective approaches to solve them [1]. They include global climate change [2], including rising temperatures, accumulation of organic waste [3,4], and pollution by toxic metals [5]. These factors have a serious impact on many species of plants, animals, and microorganisms, as well as on the relationships between their populations. In the past, the change of the habitat of a particular species of animal or plant was rare. Now, such changes are happening very quickly and unsystematically under the influence of significant man-made load and global environmental changes [6]. This has become one of the main reasons for the change in the species composition of biota, in particular the reduction of aboriginal species [7]. In this regard, such phenomena as uncontrolled outbreaks of growth of various alien monospecies populations of aquatic macrophytes have become

more frequent in the aquatic ecosystems of Ukraine [8]. *Pistia stratiotes* L. is one of the ecologically dangerous alien aquatic macrophytes.

Pistia stratiotes L. is a tropical aquatic plant that floats on the water surface. It is widespread in tropical and subtropical areas and in some places reaches mass development, forming a continuous carpet on the surface of water bodies [9]. The species *P. stratiotes*, also known as *Jalkumbhi*, is native to South America, is not a frost-resistant species, and is widely found in ponds, streams, and free-flowing rivers, mainly in tropical and subtropical regions of Asia, Africa, and America [10]. Within the traditional range, the growth of pistia is constrained by its natural enemies and local environmental conditions. But as the global climate warmed, pistia began to spread to countries with a temperate climate. Here, pistia have no natural enemies [11]. In such places, the massive growth of pistia can become uncontrolled, causing the degradation of local aquatic ecosystems and significant economic damage. The harmfulness of these plants is determined by their excessive growth in water bodies, which interferes with the photosynthesis and respiration of aquatic organisms and critically degrades water quality [12]. *P. stratiotes* L. is a free-floating plant with no odor and a bitter taste. This plant usually forms a dense mat on the surface of the water [13]. *P. stratiotes* is widespread as an ornamental plant, is often used in aquariums and garden ponds [14], is often used as a biofertilizer, and is also known as a medicinal plant [15]. However, *P. stratiotes* is able to block navigation channels, prevent fishing and transportation of boats, block the flow of water in irrigation canals, and disrupt the production of hydropower [14]. The dense plant mass of *P. stratiotes* above the water surface impairs the penetration of light and oxygen supply into water bodies and, thus, destroys their biodiversity [9]. Mechanical removal of these plants is widespread [9], but later they begin to rot on the riverbank and again contaminate ecosystems with toxic microbial exometabolites such as fatty acids an alcohols. However, the biomass of pistia is a natural carbohydrate-containing polymer [16]. Therefore, we assumed that the polymers of pistia, like cellulose and starch, could be fermented via anaerobic microorganisms with the formation of CH_4 [16]. The use of other polymeric substrates to produce biomethane is also widespread. Substrates used include meat processing waste [17], wastewater sludge [18], food waste [19], etc. However, anaerobic fermentation of aquatic plant materials is less described.

Hence, it follows that a double-positive result can be achieved with anaerobic degradation of the biomass of pistia: fast and efficient degradation of the ecologically hazardous biomass of pistia and the synthesis of the energy carrier CH_4. Pistia appeared in the water bodies of Europe in the second half of the 20th century. It was first recorded in the canals of the Netherlands in 1973 [20,21], where a periodic active growth of plants in the summer was detected in subsequent years [22]. In 1998, a massive proliferation of pistia in the waters of northern Italy was recorded [23,24]. Now, it is widespread throughout Italy [23]. In Europe, pistia is found in the Czech Republic [25], Slovenia [20], Serbia [26], and in some other countries. In recent years, pistia has been found in the Great Lakes region of North America [27]. The current distribution of this plant is so global that in some EU countries it is even forbidden to use it as an aquarium plant [28]. The intensive spread of pistia in the water reservoirs of Ukraine is greatly facilitated by its popularity among aquarists and landscape designers [29]. The rapid spread of *Pistia stratiotes* has also been observed in Serbia, especially in the Bega River, which has connections with a fairly large part of the country's river network. It is believed that the appearance of *P. stratiotes* is associated with its spontaneous spread from neighboring Romania, where the plant has been recorded for about ten years [26]. In Egypt, *P. stratiotes* occurs in the slow-flowing canals of the northern Nile Delta and reaches the city of Embaba near Cairo, as well as in calm and still waters, especially around the city of Fariskur. It was recently recorded in Lakes Mariut and Mansala in the north of the Nile Delta [30].

Although its specimens usually do not survive the winter and do not create a stable population, the situation may change due to climate change. In Ukraine, the first findings of pistia were registered near Kyiv in 2005. Since then, it has repeatedly occurred here in

natural reservoirs, but did not reach mass development [31]. In 2013–2014, a mass outbreak of pistia was registered on the Seversky Donets River in the vicinity of Kharkiv [32]. This outbreak caused economic problems (restrictions on shipping, recreation, and fishing), and more than 6 million UAH were allocated from the regional budget to fight pistia alone. The problem of spontaneous distribution of invasive aquatic macrophytes, in particular *P. stratiotes*, is relevant in Ukraine. This is due to climate change and rising temperatures in the winter [8]. Rapid distribution of the species in the lowlands of the Dnister River and in the Dnipro and Siversky Donets Rivers has been reported [33]. Single specimens of the plant were found both in the city hydrographic network and in the shallow water of the Dnipro River near Kyiv [34]. It has been experimentally confirmed that after the removal of invasive freshwater plants, including *P. stratiotes*, the biodiversity of water bodies is restored [35]. Various methods are used to control these weeds in aquatic environments (e.g., preventive, mechanical, biological, and chemical). The most widespread method is mechanical collection. However, mechanical removal of plants leads to the accumulation of a huge amount of wet weed biomass. In most cases, it is disposed of in wastelands or burned after drying. An alternative is the use of waste in other industrial processes [9].

Since the significant growth of pistia has led to a number of economic and environmental problems, the issue of combating this phenomenon, as well as its consequences, is relevant. World experience shows the mechanical removal of pistia from the reservoir to be the most effective method (Global Invasive Species Database). This method requires fairly resource-intensive technology [36]. Pistia biomass, however, can be seen as a valuable raw material, the effective use of which can not only reduce the cost of combating this invasive species, but also bring profits. Microbial degradation to produce biogas is one of the useful applications of plant biomass [16,37], which can be used on farms. Its incineration to convert into electricity is another application, but a more hazardous one [38]. Fermentation of aquatic plant material can be accompanied by the formation of biohydrogen or biomethane. The biomethane yield was quite high at 103–262 NmL CH_4/g VS during the fermentation of beach-cast seagrass wrack [39]. Aqueous hyacinth is also a hazardous plant that can be successfully used to produce biomethane [40]. A huge CH_4 yield was obtained—337 NL/kg VS_{added}—via its cofermentation with household waste [41]. The aquatic plant *Landoltia punctate* is a substrate for the production of biohydrogen, the yield of which can reach 2.14 mol H_2/mole of reduced sugar [42]. Thus, we consider aquatic plants to be promising renewable substrate for fermentation with biogas obtaining.

Another serious environmental problem in agricultural and industrialized countries is the pollution of natural biogeocenoses with toxic metals [43]. Copper compounds are one of the most common and environmentally hazardous pollutants [44]. The main sources of copper pollution are the uncontrolled use of copper-containing fungicides and pesticides [45], deposits and industrial mining [46], as well as industrial wastewater [47]. Copper in trace concentrations is a necessary trace element for the functioning of living organisms [48]. However, when its concentration increases, it is a very toxic metal. Copper, as a catalytically active metal, is able to influence numerous metabolic, functional, and regulatory pathways in living organisms. The mechanism of the negative action of the metal is to inhibit the functioning of a number of vital enzymes—acetylcholinesterase, succinate dehydrogenase proteases, lipases, and glucosidases [49]. The gradual accumulation of copper in agricultural soils leads to an increase in their phytotoxicity and absorption copper in agricultural crops. Widely used methods of purification of contaminated ecosystems from copper compounds include physicochemical methods: adsorption [50], membrane filtration [51], cementation [52], photocatalysis, and electrodialysis [53]. These methods are cost-effective but, in some cases, do not guarantee complete remediation of contaminated ecosystems [54]. In recent decades, there has been considerable interest in methods of bioremediation of contaminated ecosystems that are based on the use of microorganisms as major biotechnological agents [55]. The microbial pathways of copper removal are based on their accumulation, precipitation (to $Cu(OH)_2\downarrow$, $CuCO_3\downarrow$), as well as reduction to insoluble and nontoxic compounds ($Cu_2O\downarrow$) [56]. Transformation reactions of divalent

copper compounds occur due to the release of microbial exometabolites both inside and outside the cells [57,58]. These associated reactions are clearly described in the method section. A number of studies have shown that microorganisms have the genetic potential to remove heavy metals from the environment [59]. The ability to transform copper compounds is determined by genetic determinants [58]. Thus, *cop* genes, chaperones, transporters, and sequestering molecules together encode copper removal mechanisms in different microorganisms [60]. Microbial biotechnology is economically viable because cheap organic substrates, including environmentally hazardous organic waste, which also includes the aquatic plant *Pistia stratiotes* L., can be used as a substrate for the growth of microorganisms [16]. Such promising integrated biotechnological approaches based on the simultaneous solution of several environmental problems are poorly studied and require significant fundamental and applied research. Thus, in this work we considered the possibility of using microbial preparations to provide a double-positive environmental effect—the degradation of the environmentally hazardous plant *Pistia stratiotes* L. and detoxification of hazardous copper compounds.

2. Materials and Methods

2.1. Production of the Granular Microbial Preparation

The method used to produce the granular microbial preparation (GMP) was as follows. The biomass of biomethane-synthesizing microorganisms was sampled in the first stage. Digested sludge from methane tanks at the Bortnytsia aeration station in Kyiv, Ukraine, was collected to be used as a source of methanogenic microorganisms. One liter of digested sludge was then mixed with the starting substrates and regulators of microbial metabolism to manufacture the granular microbial preparation in the second stage. The granules were made by extrusion using a miniextruder.

After forming, the granules were dried in a ventilated laboratory electric furnace at 40 °C to preserve the cells of methanogenic microorganisms. The GMP2 modification containing living methane-synthesizing microorganisms was then ready to use (Figure 1).

Figure 1. General view of the granular microbial preparation.

Preparation of the granules of hydrogen-synthesizing microorganisms (GMP1) is described in our previous work [61]. The preparation procedure for this type of GMP was similar to GMP2. The main difference was drying in a ventilated laboratory electric furnace at 105 °C after forming the granules. This temperature was used to destroy methanogens and select only spore-forming hydrogen-synthesizing microorganisms.

2.2. Sampling and Preparation of Substrate for Anaerobic Fermentation

Plants of *Pistia stratiotes* L. were used as a substrate for degradation via hydrogen-synthesizing (GMP1) and methanogenic (GMP2) microbial preparations.

Samples of aquatic plants were collected from Zoloche Lake (Kyiv region, Ukraine) in October 2020 (Figure 2A). For selection, the generally accepted technique of floristic and geobotanical research of features of formations of the higher water vegetation on reservoirs was used: establishment of features of overgrowth of various sites of reservoirs with the subsequent mapping of a vegetative cover of a reservoir. The biomass was collected during the expedition trip using the trial plots method and the ecological-coenotic profile method [62,63]. Plants were selected only with a satisfactory physiological condition without signs of slowing of vegetation or death. The plants were dried at 105 °C to a constant weight. Plants were dried to kill the most viable microorganisms to avoid the effect of their own microbiome on the experimental fermentation process and distortion of the results. The dried plants were ground (the size was 0.5–2.0 cm) before loading in sealed glass jars to ferment (Figure 2B).

Figure 2. The view of the *Pistia stratiotes* L. plants on the sampling site (**A**) and after drying (**B**) before loading into the sealed glass jars.

2.3. Description of the Fermentation Process

The fermentation of plants was carried out under three conditions: without GMP and with the methanogenic and hydrogen-synthesizing types of GMP to compare the effectiveness of the fermentation process.

To study the dynamics of pistia fermentation, 5 g of samples dried at 105 °C and 400 mL of boiled tap water were loaded into sealed glass jars with a total volume of 500 mL (Figure 3). The initial gas phase was air.

In one case, no GMP was added to the sealed glass jars to check the possibility of spontaneous fermentation of plants by pistia's own microbiome (Figure 3A).

In the other two cases, the samples were dried and treated with 85–90 °C tap water and cooled. Afterward, 2 g of the hydrogen-synthesizing GMP or methanogenic microbiome GMP were loaded into sealed glass jars (second (Figure 3B) and third (Figure 3C) cases, respectively). The bioreactor was closed with rubber stoppers with fittings to sample the aliquots of culture fluid and gas and to remove the synthesized gas. The synthesized gas flowed through the gas controller to the gas holder. The fermentation cycle took place over 76 days at 30 °C.
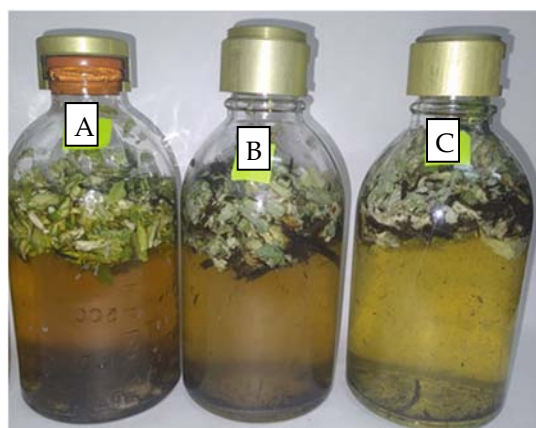
Figure 3. General view of the sealed glass jars with a total volume of 500 mL: (**A**) control variant without preparations; (**B**) with GMP1 modification containing living hydrogen-synthesizing microorganisms; (**C**) with GMP2 modification containing living methane-synthesizing microorganisms.

The following parameters were monitored: pH, redox potential (Eh), gas volume [64], and the concentrations of H_2, O_2, N_2, CO_2, and CH_4 in the gas phase, as well as total carbon [65] and ammonium ion (NH^{4+}) concentration [66] in the culture fluid.

The completion of the process was evidenced by the stabilization of pH and increase in the redox potential of the medium, termination of gas synthesis, and visual degradation of solid waste particles [67]. The experiment was carried out in triplicate (*n* = 3). Standard deviation (SD) and average values (x) were calculated.

2.4. Measurement of the Dynamic of Toxic Copper Bioremoval during the Fermentation of P. stratiotes L. Plants

The most efficient fermentation process was selected after completion of all fermentation cycles with H_2-producing and CH_4-producing types of GMP to determine the possibility of toxic Cu^{2+} microbial removal. A variant with GMP2 based on a methanogenic microbiome was used. The substrate preparation methods were identical to those described in Section 2.3.

A solution of 60,000 ppm Cu^{2+} was used as the stock. It was prepared by $CuSO_4 \cdot 5H_2O$ dissolving in distilled water in a volumetric flask. Citrate was used as a chelator. The Cu(II) concentration was determined by using the colorimetric method with 4-(2-pyridylazo)resorcinol (PAR) as well as the method of substituent titration with PAR and ethylenediaminetetraacetic acid (EDTA) in the ranges of 0.5–7.0 ppm and 25.0–3000.0 ppm Cu^{2+}, respectively. The methods are based on the property of PAR to form colored red complexes with cations of bivalent heavy metals, including Cu^{2+} [68].

The copper(II) citrate solution was added to the sealed glass jars when microorganisms reached the stage of active metabolic activity (49 day into the fermentation). This was estimated by the increase of biomethane synthesis (concentration of CH_4 in the gas phase of bioreactor increased to 60%) and the decrease of the Eh (in this experiment, Eh was decreased to −30 and −70 mV). Afterward, the copper solution was added to the bioreactor with the final concentrations 100 ppm and 200 ppm Cu^{2+} in the citrate forms, respectively. The experiment was carried out in triplicate (*n* = 3). Standard deviation (SD) and average values (x) were calculated.

2.5. Measurement of the Fermentation Parameters

The redox potential (Eh) and pHof the medium were measured potentiometrically. For this purpose, measurement of pH and Eh was performed using the EZODO MP-103 universal ionomer with the remote electrodes and temperature sensor. To measure pH and Eh, we used the combined Ezodo ceramic chloride electrodes with BNC connectors—PY41 and PO50 models, respectively. Before the measurement, the electrodes were tested by standard buffer solutions. For the pH check, standard pH buffer solutions were used:

a solution of $KHC_2O_4H_2C_2O_4 \cdot 2H_2O$ (pH= 1.68), a mixture of NaH_2PO_4 and K_2HPO_4 (pH = 6.86), and $Na_2B_4O_7 \cdot 10H_2O$ (pH = 9.18). Standard pH buffers were prepared according to the producer's manual (OJSC "Kiev plant RIAP"). For the validation of Eh measurements, three redox buffer solutions were used. First—ferricyanide, with Eh = +273 mV (13.5 g/L $K_3[Fe(CN)_6]$ and 3.8 g/L $K_4[Fe(CN)_6] \cdot 3H_2O$), second—Fe(II) citrate (10.0 g/L, with Eh = −150 mV), and third—Ti(III) citrate (15.0 g/L, with Eh = −440 mV) [69].

The gas synthesis was determined by the volume of water squeezed from the gas holder into the intake manifold under the pressure of the synthesized gas.

The H_2 and CH_4 concentration was determined by using the standard gas chromatography method [64]. The chromatograph was equipped with two steel columns: I—for the analysis of H_2, O_2, N_2 and CH_4 and II—for the analysis of CO_2. Column parameters were: I—l = 3 m, d = 3 mm, with molecular sieve 13X (NaX); II—l = 2 m, d = 3 mm, with Porapak Q carrier; column temperature +60 °C, evaporator temperature +75 °C, detector temperature (60 °C), detector current—50 mA. The carrier gas was argon and the flow rate of the gas was 30 cm^3/min. The concentration of H_2 was calculated by the peak squares of its components.

To determine the concentration of soluble organic compounds by the content of the total carbon in the medium, the permanganate method was used [65]. It is based on the oxidation of carbon compounds by the potassium permanganate in strong acidic conditions. The mixture of an aliquot of the culture fluid (1 mL) with 0.1 mL of concentrated sulfuric acid was heated in a boiling water bath. The aliquots (0.1 mL) of 0.1% potassium permanganate solution were gradually added until a steady light pink color appeared. This indicated the completion of the process of carbon compound oxidation by MnO_4^-. Carbon concentration was determined according to standard calibration curves.

2.6. Calculation of the Fermentation Parameters of the Efficiency of Fermentation

The evaluation of the efficiency of the process of the fermentation of the *Pistia stratiotes* L. plants was determined by the following parameters:

- waste degradation time (T, days)—defined as the duration of the process from the moment of the fermentation start until its termination (the termination of gas synthesis, etc.),
- molecular hydrogen yield—calculated as the amount of H_2 (L) synthesized from 1 kg of waste counting to the volatile solids (VS),
- biomethane yield—calculated as the amount of CH_4 (L) synthesized from 1 kg of waste counting to the volatile solids (VS),
- coefficient of waste degradation (Kd)—the degree of waste digestion. For pistia plants, it was calculated as the ratio of initial and final weight of waste counting to the VS: Kd = m_1:m_2 (where m_1 is the initial weight of dry waste; m_2 is the weight of dry detritus). To determine the initial weight of waste, it was dried to a constant weight at 105 °C and weighed. To determine the weight of detritus, the non-fermented residues after fermentation were washed in distilled water, in the solution of weak organic acid, and again in distilled water. The washed residues were dried to a constant weight at 105 °C and weighed.

2.7. Theory/Calculation

Thermodynamic prediction was applied as the theoretical background to predict the possibility of citrate complex of Cu^{2+} removal from the solution by anaerobic microorganisms. The thermodynamic prediction allows for theoretically substantiating the most effective mechanisms of soluble toxic copper(II) ion detoxification by microbial reduction to insoluble copper(I) in the form of $Cu_2O\downarrow$ or precipitation of Cu^{2+} in the forms of $Cu(OH)_2\downarrow$, $CuCO_3\downarrow$, etc. [70].

In accordance with our prediction, microbial metabolism is possible only in the zone of thermodynamic stability of water, i.e., in the range of values of the standard redox potential (E_o') from −414 to +814 MB [71]. The standard redox potential of the reaction

$2Cu^{2+} + 2e + H_2O = Cu_2O\downarrow + 2H^+$ is equal to +380 mV at low concentrations (equal or less than 0.01 M Cu^{2+}). Consequently, the redox potential of this reaction is located inside the zone of thermodynamic stability of water (from −414 to +814 mV). Therefore, reaction of Cu^{2+} reduction to insoluble copper oxide(I) is theoretically premised to be carried out by microorganisms. We consider metal reduction by microorganisms as a binary redox reaction. In this reaction, metabolically active microorganisms are the donor system, and high-potential compounds of copper(II) are the acceptor system. It is obvious that the efficiency of metal reduction is proportional to the potential difference between the acceptor and donor systems. It follows that microorganisms with the lowest values of the redox potential must reduce copper(II) with maximum efficiency. Strict anaerobic bacteria are known to create the lowest redox potential ($E_o' = -414$ mV). The redox potential of the Cu^{2+} reduction $Cu^{2+} + H_2O + 2e = Cu_2O\downarrow + 2H^+$ is +380 mV (pH = 4.6). It is obvious that the potential difference in 794 mV between the acceptor and donor systems will ensure the fastest possible reduction of soluble copper(II) compounds to insoluble $Cu_2O\downarrow$. From this background it follows that only low-potential strict anaerobes, including biohydrogen- and biomethane-producing microbial communities, are the most effective in removing copper compounds from solutions.

Microbial removal of Cu^{2+} compounds from a solution is possible not only due to the reduction reaction but also as a result of substitution (precipitation) reactions. Firstly, nontoxic insoluble copper hydroxide $Cu(OH)_2\downarrow$ can precipitate, owing to the biologically mediated increase of the medium pH up to 4.5–5.0. Secondly, the Cu^{2+} cation can interact with the CO_3^{2-} anion and form insoluble copper carbonate $CuCO_3\downarrow$ in neutral and slightly alkaline conditions: $Cu^{2+} + CO_3^{2-} = CuCO_3\downarrow$ [56]. Thus, all metabolic pathways of microorganisms interacting with copper(II) can be theoretically justified via the thermodynamic prediction method to provide the environmental effect of the purification of contaminated solutions and industrial sewage from soluble toxic Cu(II) compounds. Methanogenic microorganisms as the components of the microbial communities of natural and man-made ecosystems are strict anaerobes. In natural microbiocenoses, they function simultaneously with sulfate reducers, which are able to provide them with a source of reduced sulfur (S^{2-}). Many species of methanogenic microorganisms do not have the enzyme dissimilation sulfate reductase, and some are able to synthesize hydrogen sulfide (H_2S) via dissimilation sulfur reduction in the presence of free sulfur S^0 as an electron acceptor and conventional energy substrates (H_2 or methanol) as electron donors. Thus, in the process of biomethane fermentation, a significant amount of hydrogen sulfide is released, which precipitates sulfides of divalent metals, in particular Cu^{2+}, due to the reaction $2Cu^{2+} + H_2S^{2-} \rightarrow Cu^2 + S^{2-}\downarrow + 2H^+$ [72]. That is why the metabolic pathway of biomethane fermentation of organic substrates can be a highly efficient pathway of microbial removal of toxic and soluble copper compounds from solutions.

3. Results

We registered a significant growth of pistia near Kyiv, in the system of lakes and canals connected to the discharge channel Bortnytska aeration station. At the end of summer, a pistia carpet covered a number of reservoirs, in particular, Lake Zoloche near the village of Vyshenky, the mirror coverage of which reached 90%. The pistia biomass in the cluster reached 10 kg/m^2, the total pistia biomass in the lake was about 2000 tons.

Three variants of *P. stratiotes* L. plant fermentation were investigated. The process of fermentation without the addition of GMP was simulated in the first case. In the second and third cases, the plants were fermented with granular microbial preparations as inoculum (biohydrogen and biomethane-synthesizing microorganisms, respectively). The basic fermentation parameters of plant degradation were obtained (Figure 4). The initial values of pH, redox potential, concentration of organic compounds and ammonium ions were approximately the same. Thus, the initial pH in all variants of the experiment ranged from 7.14 ± 0.4 to 7.18 ± 0.3 and Eh from +303 ± 11 to +329 ± 18.2 mV. The concentration of NH4$^+$ ranged from 17.2 ± 8.3 to 23.1 ± 11.5 ppm and the concentration of soluble organic

compounds was 30 ± 14.5 to 41 ± 12.1 ppm (Figure 4). In all vials at the beginning of fermentation, the gas phase was air and consisted of O_2 (from $20 \pm 2\%$ to $22 \pm 2\%$), N_2 (from $77 \pm 7\%$ to $80 \pm 5\%$), and CO_2 (from $0.15 \pm 0.05\%$ to $0.22 \pm 0.1\%$) (Figure 5).
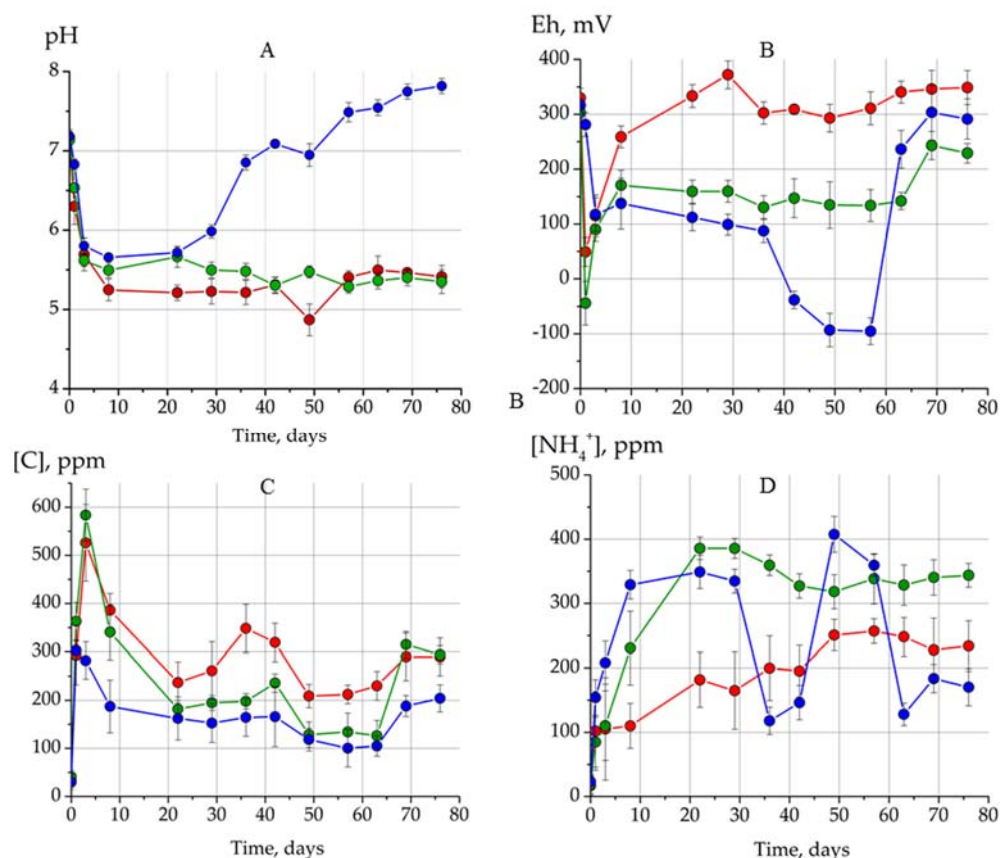
Figure 4. The main metabolic parameters of *P. stratiotes* L. plant fermentation: pH (**A**); Eh (**B**); concentration of total carbon (**C**); concentration of ammonium ions (**D**). The graph shows the dynamics of changes in metabolic parameters in control conditions without GMP (red lines), in the presence of GMP1 based on hydrogen-synthesizing microorganisms (green lines), and in the presence of GMP2 based on methane-synthesizing microorganisms (blue lines).

In the control variant of the experiment, the degradation of the plant was not observed, and the metabolic parameters of the culture fluid indicated the absence of the fermentation process (biohydrogen and biomethane were not synthesized, pH and Eh did not change significantly) (Figures 4 and 5). Thus, in the variant of the experiment without granular microbial preparation (indicated by red lines on the graphs), high values of redox potential were observed during the whole fermentation process, which ranged from 258.6 ± 20.0 mV to 348.6 ± 31.0 mV at the 8th and 76th day of fermentation, respectively (Figure 4B). After treating the fragmented pistia with boiled water, most of the aboriginal microorganisms died. However, due to the residual microbiome there was a decrease in pH from 7.2 ± 0.1 to 5.2 ± 0.2 for 8 days (Figure 4A). The presence of viable microorganisms in the control version of the experiment was also evidenced by the increase in the concentration of total carbon and NH_4^+ (Figure 4C,D). This indicates the presence of processes of hydrolysis of pistia polymers and the accumulation of liquid organic compounds (products of hydrolysis) in the culture medium. Despite this, the fermentation process was insignificant, the synthesis of excess biohydrogen and biomethane was not observed, and the basis of the gas phase was CO_2 and N_2 (Figure 5B,C). The initial gas composition of the sealed glass jars was air. Air does not inhibit the anaerobic degradation of the plants. The presence of O_2 in the air, on the contrary, stimulates the growth of aerobic microorganisms. Aerobic microorganisms

in a sealed jar completely consume O_2 and reduce the redox potential in a liquid medium. Such conditions are suitable for the growth of strict anaerobic microorganisms.
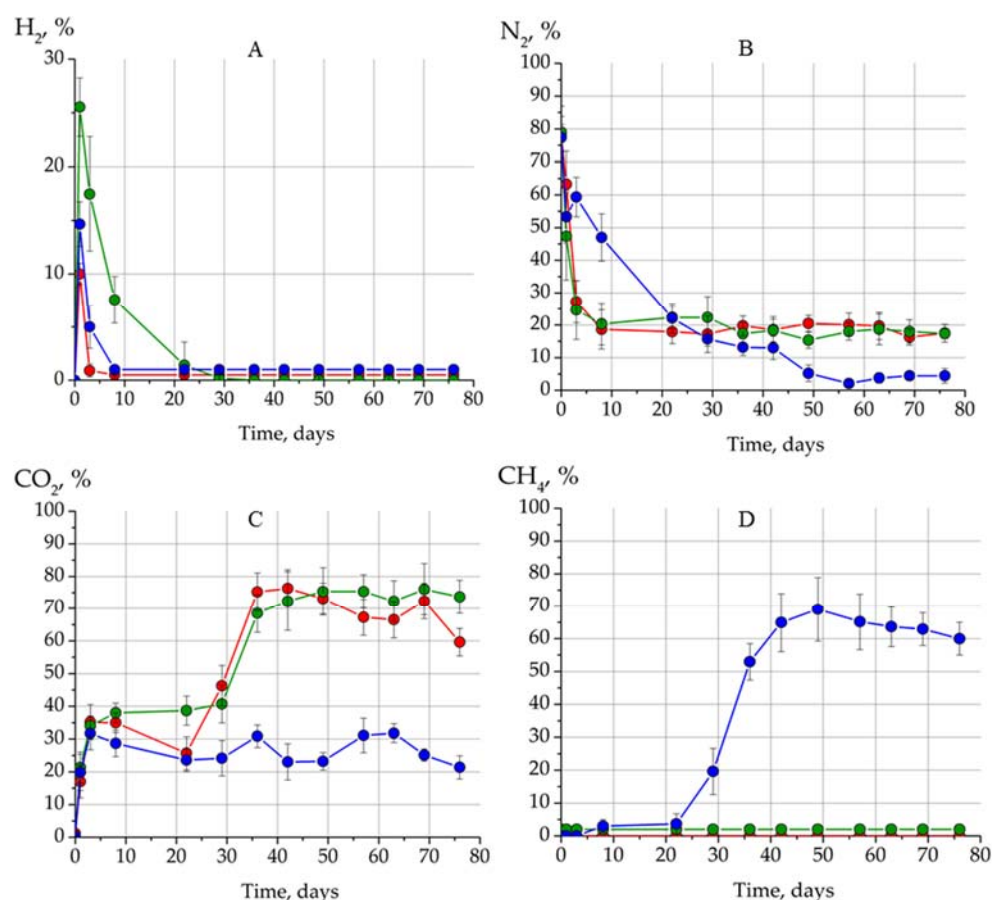
Figure 5. The composition of the gas phase ((**A**)—H_2; (**B**)—N_2; (**C**)—CO_2; (**D**)—CH_4) during fermentation of *P. stratiotes* L. plants in control conditions without GMP (red lines), in the presence of GMP1 based on hydrogen-synthesizing microorganisms (green lines), and in the presence of GMP2 based on methane-synthesizing microorganisms (blue lines).

Thus, the concentration of CO_2 in the gas phase reached $75.2 \pm 5.8\%$, while the N_2 content was $19.7 \pm 3.0\%$ on day 36 of fermentation (Figure 5B,C). Hydrogen appeared in the gas phase on day 1 of cultivation ($10.0 \pm 1.3\%$), but later its concentration decreased to absolute zero (Figure 5A). Oxygen was completely transformed by microorganisms in all variants of the experiment within 1 day of fermentation, so its illustration on the graphs was impractical. In the first case, pistia was inaccessible to aboriginal microorganisms, and the fermentation process was inefficient. As a consequence, after 76 days of fermentation *Kd* was 1.3, and CO_2 yield was 2.7 ± 0.4 L/kg of plants.

Patterns similar to the control conditions (without GMP) were observed in the second case of the experiment (in the presence of GMP1). Thus, pistia plants were also poorly accessible for degradation via hydrogen-synthesizing microorganisms. The main metabolic parameters of the second variant of the experiment are presented in the form of green lines in Figures 4 and 5. The process of degradation under such conditions was also inefficient (*Kd* = 1.5). The H_2 yield was only 0.4 ± 0.06 L/kg. As in the control experiment, the pH decreased from 7.1 ± 0.5 to 5.6 ± 0.1 during the first three days of fermentation (Figure 4A). The Eh decreased to -44.3 ± 19.9 mV on the first day of fermentation, but subsequently increased to positive values in the range of $+170.6 \pm 27.7$ to $+243 \pm 25.6$ mV (Figure 4B). However, in contrast to the control experiment, in the first three days there was a significant increase of the concentration of H_2 in the gas phase of the bioreactor.

Thus, the concentration of H_2 on the first day of fermentation was $25 \pm 2.73\%$, but over time it decreased rapidly—up to $17.5 \pm 5.31\%$ on the 3rd day and up to $7.6 \pm 2.2\%$ on the 8th day (Figure 5A). During the next two weeks, the hydrogen concentration was at a level of 1.4 ± 0.9 to $2.0 \pm 0.7\%$ and then disappeared altogether. Excess gas synthesis was not observed in either the control version without GMP or in the presence of hydrogen-synthesizing granular microbial preparation. The CO_2 concentration increased moderately during the first month of fermentation and subsequently doubled sharply from 40.7 ± 5.8 (29 days of fermentation) to $72.3 \pm 9.1\%$ (42 days of fermentation) (Figure 5C). Active hydrogen synthesis on the first day of cultivation and subsequent cessation of its synthesis and the fermentation process in general may indicate rapid depletion of available substrate for hydrogen-synthesizing microorganisms during the first days of cultivation and the absence of enzyme systems for hydrolysis of *P. stratiotes* L. plants in GMP1.

The granular microbial preparation based on methanogenic microorganisms (GMP2) was used as an inoculum in the third case. In this variant, an efficient methane fermentation of pistia and a high level of its degradation were observed ($Kd = 9$, CH_4 and CO_2 yields were 68.0 ± 11.1 L/kg VS and 43.0 ± 6.7 L/kg VS of plants, respectively). The dynamics of the fermentation process are shown in Figures 4 and 5 by blue lines. Thus, the degradation of pistia plants began with the participation of aerobic microorganisms, as evidenced by the decrease in the concentration of O_2 in the gas phase from 21.4 ± 3.8 to $0.05 \pm 0.01\%$ within one day. Subsequently, the degradation of polymers occurred via hydrogen fermentation with the formation of gaseous metabolites such as H_2 and CO_2. Hydrogen synthesis began on day 1 of fermentation and its concentration in the gas phase was $14.6 \pm 2.1\%$. On the second day, there was a decrease in the H_2 synthesis ($5.0 \pm 1.1\%$), and on the third day synthesis stopped (Figure 5A). The methanogenic microbial preparation was able to autoregulate the metabolic parameters of the medium to optimize for its growth. As a consequence, the pH level decreased only to 5.8 ± 0.8 on the third day of fermentation, but on day 29 it was 6.0 ± 1.1 and on day 42 it increased to 7.1 ± 1.4. At the end of the fermentation process, which lasted 76 days, the pH was 7.8 ± 0.1 (Figure 4A). The decrease of the redox potential from 316.3 ± 32.5 to -93.3 ± 30.5 mV occurred on the 49th day of cultivation and correlated with the increase of the concentration of CH_4 in the gas phase of the bioreactor (Figures 4B and 5D). The concentration of biomethane on the 50th day of fermentation was very high—$69 \pm 9.8\%$. This indicates that GMP2 contained a diversified microbial community based on both methanogens and other types of microorganisms—destructors of plant polymers. CO_2 synthesis occurred throughout the experiment and did not exceed $31.8 \pm 2.8\%$ (Figure 5C). At the same time, the content of soluble organic compounds (determined by total carbon) and the content of $NH4^+$ (Figure 4C,D) in the culture fluid increased, which also indicates the degradation of plant polymers to liquid compounds (Figure 4C,D).

Usually during the degradation of plant polymer compounds (for example, starch) there is an intensive synthesis of gas, increasing its volume and increasing the pressure in the hermetic bioreactor to 1.0–1.5 atm. However, for pistia fermentation, an increase in gas volume was registered only in the GMP2 case. This was apparently due to the low content of available carbon-containing compounds in pistia tissues for the aboriginal pistia microbiome of GMP1 and the high availability of plant polymers for GMP2. Microorganisms of GMP2 rapidly and effectively destroyed the pistia. Visually, it was the degradation of the plants was noticeable on the 3rd day and appeared to end completely on the 50th day. However, the fermentation of available substrates occurred for another month after complete visual degradation of the pistia. The decrease in pistia weight was determined by the degradation coefficient Kd, which is equal to the ratio of the initial and final mass in terms of volatile solids. The initial dry weight of pistia was 5.0 g, and after fermentation it was 0.55 g, so $Kd = 9$. Thus, it was shown that pistia degradation was carried out with the production of the energy carrier biomethane. This preparation can be used for bioremediation of waters overgrown with alien pistia plants. At the first stage—removal and collection of pistia biomass from reservoirs, and at the second—its

fermentation in a bioreactor with effective weight reduction and obtaining the high energy carrier CH_4.

The last stage of the work was the study of the dynamics of the removal of soluble compounds of Cu^{2+} in citrate form by GMP2 based on methanogenic microorganisms. The third variant of the experiment (with GMP2) was chosen to model the process of copper detoxification, as only this variant of the experiment was highly effective in the degradation of pistia plants. This part of the research was carried out for several purposes. The first was to confirm the thermodynamic position that methanogens are able to remove copper compounds from solutions. The second was consideration of the degradation of an aquatic plant as an integrated process that can be used for both obtaining biofuel and detoxification of common metals. The copper solution in the form of Cu^{2+} citrate was added in the active phase of fermentation at 49 h of cultivation to final concentrations of 100 ppm and 200 ppm, respectively (Figure 6A,B). In both variants, after the addition of the copper solution there was a sharp increase in the redox potential. The Eh ranged from -86 ± 31.7 mV to $+286 \pm 28.5$ mV after the addition of 100 ppm Cu^{2+} (Figure 6A) and from -16.3 ± 10.1 mV to $+290 \pm 22.2$ mV after the addition of 200 ppm of Cu^{2+} (Figure 6B).
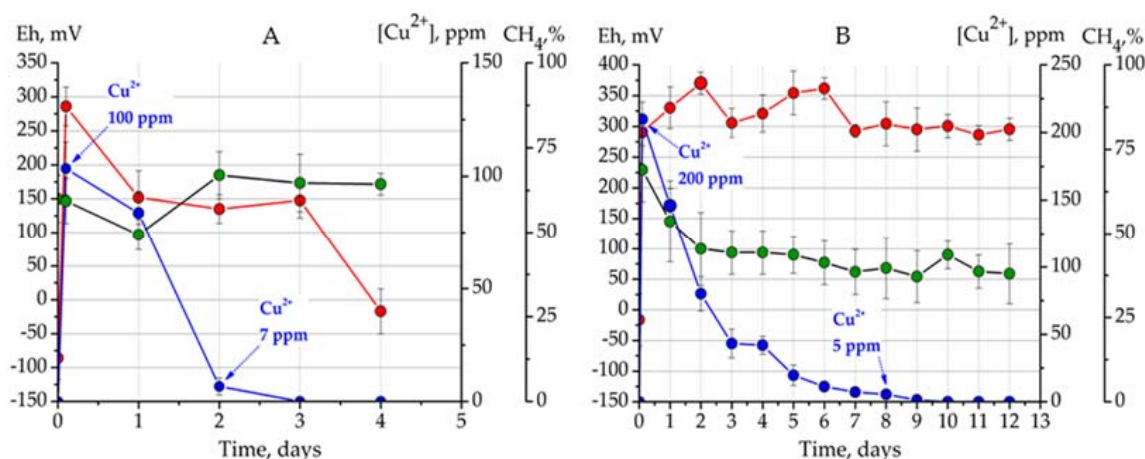
Figure 6. Effective removal of toxic copper compounds during the fermentation of pistia plants at the initial concentrations of 100 ppm (**A**) and 200 ppm (**B**) Cu^{2+}.

Removal of 100 ppm Cu^{2+} took only three days and correlated with a slight decrease in redox potential in the medium. During the first day, there was an inhibition of biomethane synthesis, which manifested itself in a decrease in concentration from $60.0 \pm 7.0\%$ to $49.4 \pm 4.5\%$, but when the concentration of Cu^{2+} decreased after 2 days to 7.0 ± 3.4 ppm, biomethane synthesis again increased to $67.0 \pm 6.9\%$ (Figure 6A). The redox potential also gradually decreased to a more optimal level for the growth of methanogens and on day 4 was -17.0 ± 23.1 mV (Figure 6A). Copper at the concentration of 200 ppm inhibited the process of biomethane fermentation. This was manifested in the inhibition of biomethane synthesis, increasing Eh to very high and suboptimal values for methanogens in the range $+290 \pm 30.2$ to $+370 \pm 17.9$ mV (Figure 6B). Despite the suppression of the metabolic process by soluble copper(II), it was completely removed from the medium within 10 days of fermentation. The metabolic parameters did not return to baseline, and the biomethane concentration finally decreased from $69.3 \pm 7.3\%$ to $37.1 \pm 7.71\%$ (Figure 6B).

Thus, the application of GMP2 for the degradation of pistia plants reduced their concentration by 9 times, as evidenced by the coefficient of degradation of *Kd*. The duration of the cycle was 76 days, after which gas synthesis was terminated. The yield of biomethane was 68.0 ± 1.1 L CH_4/kg VS of plants. The application of GMP1 was accompanied by an inefficient process and did not contribute to the degradation of pistia plants (*Kd* = 1.5).

4. Discussion

Obtaining biogas during the fermentation of macrophytes is one of the promising areas considered in a number of studies. [16,73]. The main advantages of using aquatic plant biomass are lack of competition with food crops for arable land, high growth rates, low fractions of lignin, which reduces the need for energy-intensive pretreatment, and compatibility with the introduction of an approach to bioprocessing [74]. Aquatic *P. stratiotes* plants contain a lignocellulosic biomass, which is a mixture of cellulose, hemicellulose, and lignin, and therefore have great potential for conversion into bioenergy. The anaerobic fermentation of *P. stratiotes* via activated sludge waste as inoculum was investigated. The highest biogas yield previously obtained was 321 mL/g of solids with a biomethane content of 72.5% [74]. In this case, the high yield of biomethane may have been caused by the presence of additional substrates for methanogens in the activated sludge. In our case, the microbial preparation was used as an inoculum, not as an additional substrate.

Obtaining biogas from aquatic plants has been gaining popularity in recent decades. In the case of [75], pistachios, aqueous hyacinths, and channel grasses were used as substrates. The biogas production was only 15.4–23.65 L/kg dry weight after a 21-day fermentation period. Thus, in the absence of additional cosubstrates, the biogas yield was quite low. In our experiment, any additional substrates are absent, and the yield of biomethane was high (68.0 ± 11.1 L/kg VS).

Another promising area of bioremediation of ecosystems is the use of microorganisms, in particular anaerobics, to remove toxic metals from solutions. For example, a microbial community of sulfate-reducing microorganisms (SRM) was able to precipitate $CuS\downarrow$ in an anaerobic sulfidogenic reactor used to treat industrial wastewater from metal compounds. Sulfate-reducing microorganisms reduce SO_4^{2-} in the process of dissimilatory sulfate reduction and emit hydrogen sulfide H_2S^{2-}, which interacts with Cu^{2+} and precipitates it in the form of the insoluble sulfide $CuS\downarrow$ [76]. The possibility of using Cu^{2+} as a terminal acceptor of electrons in the process of anaerobic respiration by microorganisms of the genus *Desulfuromonas* was proved. The efficiency of the immobilization of Cu^{2+} in the form of $CuS\downarrow$ using H_2S synthesized by microorganisms reached 97.3–100.0% in the presence of free sulfur S^0 [77]. Reduction of Cu^{2+} can also occur via interaction with microbial exometabolites. The role of extracellular polymeric substances (EPS) of bacterial strains of *Shewanella oneidensis* MR-1, *Bacillus subtilis* and yeast *Saccharomyces cerevisiae* and their redox state in the reduction of Cu^{2+} under anaerobic conditions was studied. It was found that the reduction occurred for about 10 min with the participation of cytochrome *c* and EPS (substances with phenolic and amide groups) of the studied microorganisms [78]. The possibility of the reduction of Cu^{2+} to Cu^+ by autotrophic acidophiles of *A. ferrooxidans*. and *A. caldus* was demonstrated. They were also cultivated in the presence of elemental sulfur S^0 [79]. More than 23 ppm of Cu^{2+} was reduced over 24 h at an initial concentration in the medium of 100 ppm by *Pseudomonas putida* NA. Thus, the bioremoval efficiency was only 23%, which is not an effective result. A mixed bacterial culture containing microorganisms of the genera *Thiobacillus* and *Clostridium* was capable to immobilize Cu^{2+} via reduction mechanisms. The reduction efficiency was 89–92% Cu^{2+} in the temperature range 20–35 °C [80]. Thus, it was shown that in most cases complete 100% removal of copper compounds was not achieved. Copper has been found to be one of the most toxic to methanogenic microorganisms. According to recent studies, heavy metals are toxic to methanogenes *Methanospirillum hungatei* GP1 [81], *Methanospirillum hungatei* JF1, *Methanosarcina barkeri* MS, *Methanothermobacter marburgensis* and *Methanobacterium formicicum* [82] and inhibit their vital functions. However, microbial community analysis showed the presence of microorganisms closely related with *Methanobacterium* and *Methanospirillum*, suggesting that methanogenesis can coexist with sulphate reduction and metal precipitation. The presence of sulphate and heavy metals in wastewaters can affect the performance of methanogens and therefore impact energy recovery (in the form of biogas) from organic materials. We obtained similar results and experimentally proved that the methanogenic microbial preparation is able to completely remove toxic copper compounds from solutions. It is theoretically substantiated that one of

the most important mechanisms was the precipitation of copper sulfide due to the sulfate reduction associated with methanogenesis. Despite the real evidence of high efficiency of the metal removal process during methane fermentation by precipitation in the form of sulfides, these processes still remain poorly studied and require further investigations.

5. Conclusions

The obtained results showed the general opportunity to effectively use GMP2 for methane fermentation of environmentally hazardous *P. stratiotes* L. plants. GMP2 was shown to be promising for high efficiency rapid degradation of pistia and the synthesis of high energy carrier biomethane and also complete removal of toxic Cu^{2+} from solutions where it is high in concentration. Methanogenic microbiome analysis, design of industrial plants, and economic calculations will be the next important steps to develop the optimal and profitable path of simultaneous hazardous aquatic plant degradation and obtaining of biofuel.

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