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Aqueous Phase from Hydrothermal Liquefaction: Composition and Toxicity Assessment

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Abstract: The main obstacle to the widespread use of hydrothermal liquefaction (HTL) for waste and wet biomass recycling is the formation of a significant amount of highly polluted wastewaters. This paper presents an analysis of the chemical composition and toxicity of aqueous phase from the HTL (HTL-AP) of primary and secondary sludge. It was shown that HTL-AP has a high level of organic pollution (total organic carbon (TOC) = 4.2–9.6 g/dm^3, chemical oxygen demand (COD) = 7.9–14.0 g/dm^3, BOD_5 = 6.0–8.1 g/dm^3) and high biological toxicity for traditional test organisms (so that dilution ratio, ensuring the death of no more than 50% of organisms (DR_{50}), varied within 64.7–142.2 and 44.9–81.7 for *Artemia salina* and *Paramecium caudatum*, respectively). An analysis of HTL-AP composition with NMR-spectroscopy method allowed us to establish that the share of carbon in aliphatic chains was 34.05–41.82% and the content of carbon in carboxyl groups and aromatic rings was 26.42–34.44%. As a result, we can conclude that the main HTL-AP components are fatty carboxylic acids and their derivatives, aromatic carboxylic acids. The content of aldehydes, ketones, and lignin is less than 8%. Biological treatment of HTL-AP in a lab-scale aerobic reactor turned out to be successful, so average COD reduction was 67–95%. Sludge from an industrial waste water treatment plant (petrochemical sector) with a microorganism concentration of 2.7 g/dm^3 was used as inoculum. HTP-AP was diluted 1:10 with tap water. The duration of the process was 18 h.

Keywords: aerobic treatment; biotoxicity; hydrothermal liquefaction; wastewater

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1. Introduction

Hydrothermal liquefication (HTL) is commonly used to solve the problem of biomass and organic waste utilization with the simultaneous production of liquid synthetic bio-oil. One of the major problems, which holds back the widespread use of HTL, is the formation of highly concentrated wastewater [1].

A wastewater stream from the HTL of organic waste contains water-soluble organic and inorganic substances with a high content of biogenic elements (carbon, nitrogen,

and phosphorus). Short chain organic acids (acetic, formic, glycolic, and propanoic) are the most common chemical substances in the aqueous phase [2,3]. Depending on the type of feedstock, wastewater may contain nitrogen-including substances such as amides, heterocyclic nitrogen compounds, nitrites, sugars, furans, phenol, alcohols, ketones, and aldehydes [4]. The chemical oxygen demand (COD) of the aqueous phase is between 10 and 100 g/dm^3; total nitrogen is between 1 and 30 g/dm^3 [3,5], and total phosphorus is between 0.001 and 10 g/dm^3. This diverse composition of biomass results in a wide range of nutrients concentrations in HTL-AP, which are directly dependent on the physical conditions of the HTL of the feedstock [2,6].

Several studies have been devoted to the assessment of HTL-AP toxicity. For example, a nutrient medium containing 1% of the aqueous phase obtained by HTL of *Miscanthus giganteus* biomass was highly toxic for the cultivation of *Vibrio fischeri* cells, killing more than 50% of the cells. The presence of hydrocarbons, such as fluorene, phenanthrene, fluoranthene, and pyrene [7], which inhibit the growth of microorganism cells, contributes to the high toxicity. Wastewater at concentrations of 0.2% and 0.25% of sugar cane pulp liquefaction caused 100% death of microscopic fungi *Coniophora puteana* [8].

The high concentrations of pollutants, as well as a significant variation in the composition of the HTL-AP, depending on the feedstock and process conditions, indicate the importance of research in the field of wastewater composition assessment and treatment methods selection.

Oxidation methods primarily include ozonation, hydrogen peroxide (H_2O_2), ultraviolet ray treatment, and chlorination processes and are used as the first stage of HTL-AP purification prior to biological treatment, because it contains compounds capable of inhibiting the growth of algae, pure cultures of bacteria, and methane-producing microbes, according to the literature. Aken et al. [9] demonstrated that the ring structures of aromatic and heterocyclic compounds are oxidized to carboxylic acids, which can then be used as a substrate for biogas synthesis by methane-producing bacteria. Ozone pre-oxidation reduced the concentration of phenol and N-heterocyclic nitrogen compounds in the aqueous phase obtained after manure liquefaction by more than 20%. The main disadvantage of this method is that it is expensive and sensitive to pollutant concentration changes [10].

The use of wet oxidation to treat wastewater from HTL allows the mineralization of 75–97% of organic pollutants in 30–180 min at a temperature of 125–350 °C, a pressure of 0.5–20 MPa, and using oxygen as an oxidizing agent [11,12]. The main drawback of this method is the high operating costs associated with the need to maintain high temperature and pressure [12].

Traditional sorbents, such as zeolites and activated carbon, are commonly used as adsorbents for the purification of HTL-AP. The zeolite can effectively adsorb ammonia from the aqueous phase and is suitable for the pre-treatment of nitrogen-rich wastewater (obtained after HTL of activated sludge or animal manure) [13]. Zhou et al. [14] reported that the pre-treatment of the aqueous phase with activated carbon led to a COD reduction of up to 33.3% of the initial value. One of the major drawbacks of the adsorption process is the need to dispose of the used sorbents [15].

Biological methods for the utilization HTL-AP, which are divided into aerobic and anaerobic ones, come to the fore in light of the general trend toward the maximum use of biotechnological nature-like technologies [5].

He et al. reduced COD to 93.4% in the process of cultivating *Rhodococcus opacus* and *Rhodococcus jostii* pure cultures on algal HTL-AP. At the same time, *Rhodococces opacus* synthesized valuable lipids in the amount of 0.43 g/g d.m. [16].

The issue with this approach is that many compounds can inhibit the growth of pure cultures. Jayakody et al. [10] states that the genetic modification of *Pseudomonas putida* increased the resistance to the aqueous phase by a factor of 200 [10].

The anaerobic wastewater treatment method is one of the most cost-effective technologies for the treatment of highly concentrated wastewater, and it is used around the world to generate electricity, heat, and natural gas (methane). According to studies, the efficiency

of anaerobic digestion is affected by the type of feedstock and the conditions of HTL. On average, the COD reduction efficiency of water ranged from 37% (water after liquefaction of *Spirulina* sp.) to 93% (water after liquefaction of manure); the range of methane output was from 14 to 314 mL/g COD. Most studies show that the average methane yield is 200 mL/g COD, which corresponds to an energy recovery of 57% [17].

The problem of implementing anaerobic digestion and aerobic treatment is associated with the presence of compounds toxic to biota [14,18–20]. During the fermentation of HTL-AP obtained from *Spirulina*, the inhibition of microorganisms was observed when the HTL-AP dose exceeded 5% HTL-AP; complete inhibition of microorganisms was observed at a concentration of HTL-AP of 24% [21].

A biological treatment method was selected for handling HTL-AP after a comparison of the available options based on universal applicability and maximum economic efficiency. Most studies, along with the success and prospects for the implementation of this method, note the problem associated with the high toxicity of wastewater for aerobic and anaerobic biota. In light of this, it is a relevant research task to determine how wastewater from HTL varies in quality depending on the initial processed raw materials, the conditions of the process, and the presence or absence of catalysts. This study also assessed the degree of toxicity of HTL-AP based on biotesting using ciliates *Paramecium caudatum* and crustaceans *Artemia salina* as test organisms [22,23]. The main objective of the study was to determine the feasibility of using aerobic biological methods for wastewater treatment from the HTL of secondary and primary sludge.

2. Materials and Methods

2.1. Materials

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) and Helicon (Moscow, Russia). The main raw material for the HTL in this study was sludge from a municipal wastewater treatment plant in the city of Kaliningrad. The total capacity of the wastewater treatment plant is 150 thousand tons. The plant is quite typical in that it uses one-stage biological treatment in continuous-flow aeration tanks. Primary and secondary sludges were used for the research. The main physicochemical characteristics of the studied sludge are presented in Table 1.

Table 1. The main physicochemical characteristics of the studied sludge.

Parameter	Primary Sludge	Secondary Sludge
C content, %	54.58 ± 2.33	56.66 ± 0.31
H content, %	8.20 ± 0.28	8.93 ± 0.11
O content, %	25.30 ± 0.96	20.74 ± 0.18
N content, %	4.22 ± 0.25	10.22 ± 0.12
Ash content (% d.m.)	26.93 ± 0.23	31.3 ± 0.13
Total lipid content (%)	5.2 ± 0.5	10.2 ± 0.7
Total protein content (%)	9.98 ± 0.21	20.32 ± 0.21
Total carbohydrates content (%, calc.)	57.89 ± 0.26	38.179 ± 0.25
HHV, MJ/kg (d.m.)	7.41	8.69
Initial water content, %	92–95	92–98

The studied wastewater was formed during the HTL of sludge in a 300 mL autoclave reactor (Eartha Zhang's, Beijing, China) with external heating. In all experiments, the temperature ranged from 240 to 280 °C, the holding time was 10–60 min, the pressure was 3.2–6.5 MPa, and the raw material-to-water ratio was 1:10–1:20 (dry matter). The following parameters were set as standard conditions: temperature 260 °C, time 20 min, raw material-to-water ratio 1:10. Homogeneous and heterogeneous catalysts were used in some experiments.

The reactor was cooled after the process, and the biochar was separated from the liquid phase by filtration through a paper filter. Next, the liquid phase was separated using

dichloromethane into an aqueous phase and bio-oil. The separated aqueous phase was the subject of this research.

2.2. Methods of Water Composition Analysis

The total nitrogen content was determined using the Kjeldahl method and a semiautomatic system from Behr Labor-Technik™ (Düsseldorf, Germany).

Chemical oxygen consumption is an important indicator of the level of organic pollution in wastewater, which was determined using the potassium dichromate method [24].

BOD_5 was determined using the conventional titrimetric method according to ISO 5815-1:2003 [25]. Sludge from the industrial treatment facilities was used as an inoculant.

The total carbon content was determined using an automatic elemental analyzer, Elementar TOC enviro (Langenselbold, Hesse, Germany). pH factor was determined using the potentiometric method using the pH meter Ohaus ST2100-E (Newark, NJ, USA).

Due to high variability in wastewater composition, it was decided to use NMR spectroscopy to determine it. Water samples were lyophilized to a constant weight to remove water and maximize the preservation of volatile impurities and easily-destroyed compounds. The dry residue was further dissolved in deuterated dimethyl sulfoxide (DMSO-D6) and deuterium oxide (D_2O) (10% of the volume of the aqueous phase).

NMR spectroscopy was performed on a Bruker Avance III HD spectrometer (400 MHz 1H, 101 MHz 13C). The choices of conditions for the solution preparation, the methodology, and the procedure for processing experimental study results were based on the available literature data [26–28].

Two types of data were obtained—the part soluble in DMSO-D6 and D_2O. Chemical shifts were determined relative to tetramethylsilane by hexamethyldisiloxane—in DMSO-D6: 1H: 0.06 m.d.; 13C: 1.96 m.d.; in D_2O: 1H: 0.28 m.d.; 13C: 2.31 m.d. or by the residual signal of the solvent DMSO-D6 1H: 2.50 m.d.; 13C: 39.52 m.d.; temperature during measurements—40 °C.

A statistical analysis of elements content and NMR spectroscopy results was performed using the software package supplied with the devices. For other parameters, the average value was calculated from the results of three measurements, and the standard deviation was calculated using the built-in Excel Microsoft tools.

2.3. Biotoxicity Evaluation

The biotoxicity of the wastewater was determined using a three-day culture of *Paramecium caudatum* ciliates and nauplii of *Artemia salina* crustaceans, in accordance with the methods described in the literature [29,30] at a 1 h exposure time. Dilution ratio, ensuring the death of no more than 50% of organisms (DR_{50}), was calculated by plotting the death graph as described by P.Yu. Galitskaya et al. [31].

The *Paramecium caudatum* culture was grown under natural light conditions at a temperature of 21–24 °C. Dry baker's yeast was used as feed when settled (dechlorinated) water was used [22].

The incubation period was 3–4 days. Toxicity was determined by the death (immobility) of test organisms exposed to toxicants present in the aqua phase after the HTL of sludge in accordance with Equation (1).

$$T = \frac{\mathrm{a} - b}{b} \cdot 100, \quad (1)$$

where a, b represent the number of mobile ciliates before and after the end of the experiment.

Using a Pasteur pipette, a drop of culture liquid containing 8–15 ciliates in a volume of 0.02 cm^3 was placed into a series of micro wells. Then, 0.3 cm^3 of the studied wastewater was added and 0.3 cm^3 of dechlorinated tap water was added to the control well. After an hour of exposure at 20–24 °C, the number of living organisms was counted [22]. The dilution ratio, ensuring the death of no more than 50% of organisms (DR_{50}), was determined using the graphical method.

Artemia salina cysts were incubated in water containing 1% NaCl under optimal incubation conditions [23]. The toxicity assessment was based on *Artemia* mortality caused by toxicants present in the aqua phase after the HTL of sludge (2).

$$T = \frac{a}{b} \cdot 100, \quad (2)$$

where a is the initial number of organisms and b is the number of dead organisms in the tested water in 24 h.

In a flask, 10 cm^3 of tested liquid or cultivation water (as control) was poured together with four *Artemia salina*. Flasks with crustaceans were left for 48 h at a temperature of 21–25 °C. After this time, the surviving and dead individuals were counted. Immobilized individuals were classified as dead [24].

The HTL-AP was diluted 1, 10, 100, and 1000 times with cultivation water. The degree of toxicity of the aqueous phase was determined by the number of surviving test organisms (Table 2).

Table 2. Degree of solution toxicity depending on the death rate of the test organisms.

Mortality of Test Organisms, %	Solution Toxicity
0–30	Non-toxic (NT)
30–50	Low-toxic (LT)
50–100	Highly-toxic (HT)

2.4. Biological Aerobic Treatment of HTL-AP

Purification of HTL-AP after hydrothermal liquefaction of primary and secondary sludge was performed on a lab-scale biological wastewater installation comprised of a cylindrical aerotank equipped with an aeration system under conditions as close to industrial as possible. The secondary sludge from the industrial wastewater treatment plant (petrochemical sector) was used as inoculum. Microorganisms concentration in the sludge slurry was 2.7 g/dm^3. HTL-AP was preliminarily diluted with dechlorinated water to a ratio of 1:1 and the experiment lasted 18 h, which corresponds to the process time at full-scale water treatment facilities.

3. Results and Discussion

3.1. HTL-AP Chemical Composition

Table 3 presents the results of the physicochemical properties assessment of HTL-AP. According to the results of the experiments, the pH of samples varied between 6.5 and 9.3.

The sample where $CuSO_4$ was used as a catalyst has a lower pH value, which is to be expected, due to the presence of a strong anion and a relatively weak cation in the composition of the catalyst. At the same time, the use of Al_2O_3 and iron-ammonium alum as a catalyst leads to the alkalization of wastewater. Performing the process without catalysts did not lead to a significant shift in pH. According to the literature data [32], such a shift is possible with the HTL of biomass composed primarily of cellulose, because the processes of its destruction result in the formation of predominantly carboxylic acids. In the case of primary and secondary sludge, the biomass is well balanced and contains significant amounts of lipids and proteins (Table 1), therefore a pH shift to the acid side was not observed.

An examination of the wastewater quality revealed very significant fluctuations in the number of organic compounds and the degree of their oxidizability. As a result, COD levels in the analyzed wastewater ranged from 7890 mgO/dm^3 to 14,000 mgO/dm^3. BOD_5 ranged from 5955 to 8137 mgO/dm^3 (Figure 1).

Table 3. Results of physicochemical analysis of HTL-AP.

Conditions *	pH	Total Inorganic C (g/dm^3)	TOC (g/dm^3)	Total N (mg/dm^3)	COD (gO/dm^3)	BOD_5, (gO/dm^3)
SS, standard, 1:15	7.14 ± 0.35	0.937 ± 0.038	5.298 ± 0.582	97.3 ± 13.6	8.0 ± 0.9	6.7 ± 1.1
SS, standard, 1:20	7.51 ± 0.37	0.795 ± 0.028	3.323 ± 0.433	60.5 ± 8.3	8.0 ± 1.1	6.0 ± 0.7
SS, standard, 10 min	6.71 ± 0.33	0.746 ± 0.039	6.560 ± 1.021	122.0 ± 21.0	8.8 ± 1.8	8.1 ± 0.7
SS, standard, 15 min	6.52 ± 0.32	0.974 ± 0.014	6.634 ± 0.340	124.7 ± 11.6	8.0 ± 0.8	7.5 ± 0.5
SS, standard	7.03 ± 0.35	0.313 ± 0.016	9.311 ± 1.553	136.5 ± 15.7	10.0 ± 1.3	7.3 ± 0.9
SS, standard, 30 min	7.56 ± 0.37	0.436 ± 0.029	4.566 ± 0.967	158.2 ± 22.1	7.9 ± 1.0	6.6 ± 0.8
SS, standard, Zeolite	7.37 ± 0.36	0.657 ± 0.042	6.993 ± 0.078	37.8 ± 6.7	10.0 ± 0.7	8.0 ± 0.4
PS, standard, Zeolite	7.01 ± 0.45	0.565 ± 0.029	9.55 ± 0.906	83.2 ± 9.4	12.0 ± 0.6	6.9 ± 0.6
PS, standard, $CuSO_4$	6.78 ± 0.33	0.918 ± 0.056	4.171 ± 0.503	179.6 ± 11.9	12.0 ± 1.4	7.8 ± 0.9
PS, standard, $NH_4Fe(SO_4)_2$	9.28 ± 0.46	0.384 ± 0.009	9.326 ± 0.781	395.2 ± 40.2	10.0 ± 1.2	8.0 ± 1.0
PS, standard, Al_2O_3	9.03 ± 0.45	0.978 ± 0.050	7.420 ± 0.545	295.6 ± 30.3	10.0 ± 0.9	7.9 ± 1.3
PS, standard, $NiSO_4$	7.23 ± 0.36	0.918 ± 0.063	6.171 ± 1.301	240.4 ± 9.6	14.0 ± 0.5	7.9 ± 0.7
PS, standard, MoO_3 2 g	8.09 ± 0.40	0.577 ± 0.023	6.950 ± 0.745	157.5 ± 5.1	12.0 ± 1.6	8.0 ± 1.3

Note: * Here, and further when describing HTL conditions, the following abbreviations have been adopted: SS—secondary sludge, PS—primary sludge, standard—mean standard conditions (temperature 260 °C, time 20 min, raw material to water ratio 1:10). In the case of using homogeneous and heterogeneous catalysts, the names of catalysts are given. If the process was carried out under conditions different from the standard, the variable parameter is indicated separately (for example, "1:15" means that another biomass: water ratio was taken).

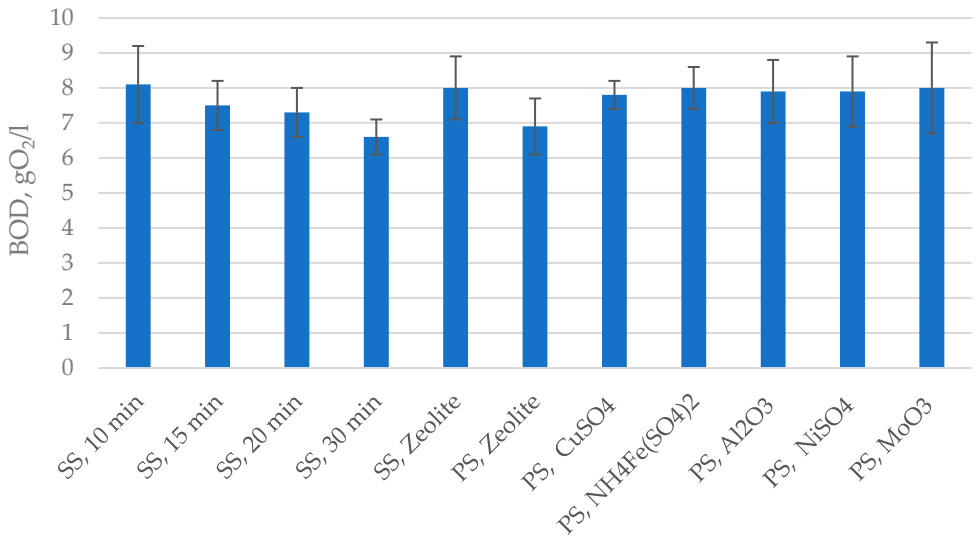


Figure 1. BOD_5 of HTL-AP.

The biodegradability of the diluted wastewater was assessed using the BOD/COD ratio (Figure 2).

Based on this ratio, it can be concluded that the minimum efficiency of aerobic biological treatment can be expected for wastewater generated during the liquefaction process using nickel sulfate as a catalyst. At the same time, water samples obtained by processing sludge without the use of catalysts, or using zeolite as a catalyst, should be expected to have a high potential degree of biological purification.

When evaluating the effect of process time on the total content of dissolved organic compounds, interesting data were obtained (Figure 3). The level of pollution with organic compounds is nearly identical when the process is performed for 10 and 15 min, but when the process is performed for 20 min, we observe a sharp increase in the level of organic pollution (by 40%), while a further increase in time leads to a decrease in organic pollution. There are currently insufficient experimental studies to explain this phenomenon. The use of HPLC-MS is likely to shed light on this issue. It can be hypothesized that the optimal time

for maximizing the degree of degradation of organic compounds, such as carbohydrates, to form soluble carboxylic acids and alcohols was exactly 20 min. Following that (with an increase in process time), this group of compounds degraded further, resulting in the formation of a gas phase (CO_2, CO) and biochar.

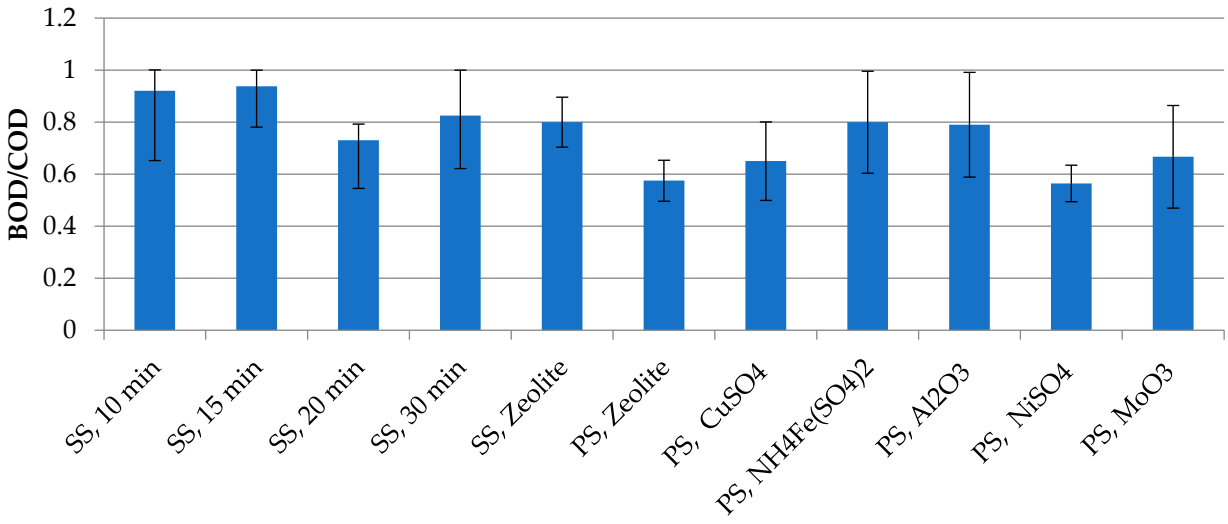


Figure 2. BOD/COD ratio.

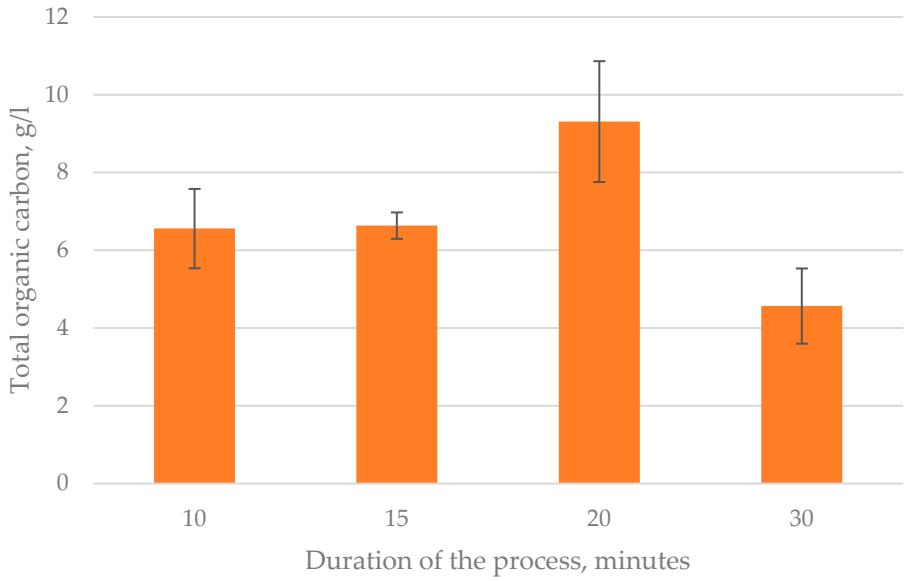


Figure 3. Dependence of HTL-AP contamination by organic compounds on the time of the process.

The maximum level of organic pollution was obtained by processing primary clarifier sludge with zeolite and iron-ammonium alum (9.55 and 9.33 g/dm^3 TOC, respectively). If we compare the level of organic pollution to a total organic carbon (Figure 4), we observe a practically identical picture as was found for the COD results. The experimental variants, in which copper and nickel sulfate were used as a catalyst, have the lowest level of organic compounds content. This could be due to the fact that these variants also have the highest yield of biooil and biochar, i.e., the use of these catalysts directs the processes of organic compound conversion to polymerization.

As mentioned above, the analysis of the composition of wastewater (some samples) was performed using the NMR spectroscopy method. The results are presented in Tables 4 and 5. In substances soluble in DMSO, carbon in aliphatic chains predominates

(34.05–41.82%), followed by carbon in bonds with carboxyl groups and aromatic rings (26.42–34.44%). As a result, we can conclude that the main HTL-AP components that are soluble in DMSO are fatty carboxylic acids (C1–C4) and their derivatives, aromatic carboxylic acids. The content of aldehydes, ketones, and lignin is less than 8%. When considering the composition of substances soluble in D_2O, a similar picture emerges.

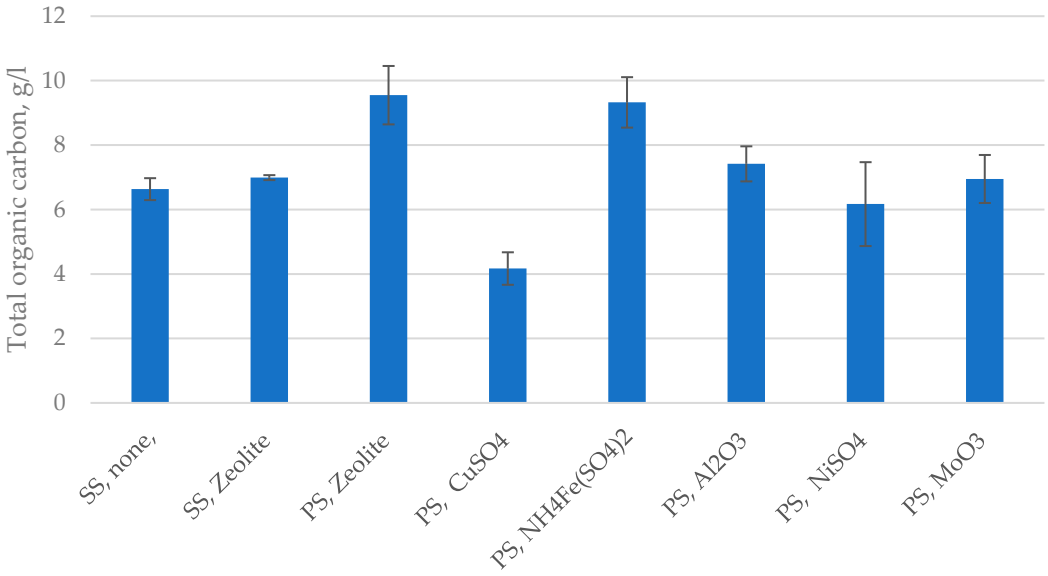


Figure 4. Dependence of HTL-AP contamination by organic compounds on the type of sludge and process conditions.

Table 4. Content of the main groups of substances (in mass %) in HTL aqueous phase produced by different types of raw materials (based on NMR spectroscopy, 1H analysis).

Conditions/Raw Material	COOH, CHO, ArOH (8.2–12.0)	Aromatic Hydrocarbons, Alkynes (6.0–8.2)	R-OH, $-CH_2-O-R$, Alkenes (4.2–6.0)	$R-CH_2-O-R$, CH_3-O-R (3.0–4.2)	$R-CH_2-CH=O$ (2.0–3.0)	Aliphatic-H (0–2.0)
			DMSO			
PS, standard, $CuSO_4$	4.22	27.77	3.01	16.15	16.81	32.04
PS, standard, $NiSO_4$	4.12	23.89	4.39	14.1	16.32	37.18
PS, standard, $CuSO_4$	0.21	26.42	3.00	18.6	13.07	38.7
SS, standard, 20 min	0.75	34.44	4.28	15.23	14.66	30.64
PS, standard, Zeolite	1.42	29.41	3.02	15.57	15.29	35.29
			D_2O			
PS, standard, $CuSO_4$	1.31	12.88	29.15	3.96	17.42	35.28
PS, standard, $NiSO_4$	2.80	22.72	8.23	24.53	15.2	26.52
PS, standard, $CuSO_4$	2.41	13.15	1.02	27.91	22.67	32.84
SS, standard, 20 min	2.02	8.03	0.2	39.75	22.58	27.42
PS, standard, Zeolite	1.58	7.77	1.92	24.48	27.97	36.28

3.2. Evaluation of the Integral Toxicity of the Aqueous Phase Using Biotesting

The toxicity assessment results are presented in Table 6 and Figure 5. Based on the experimental results for determining the dilution ratio, ensuring the death of no more than 50% of organisms (DR_{50}) for *Paramecium caudatum* ciliates (see Figure 5), the DR_{50} ranged from 44.9 to 81.7. The sample with nickel sulfate had the highest toxicity. This fact is clearly associated with the toxic effect of nickel [33]. The samples with zeolite as a catalyst were the safest.

Table 5. Content of the main groups of substances (in mass %) in HTL aqueous phase produced by different types of raw materials (based on NMR spectroscopy, 13C analysis).

Type of Raw Material, Conditions	Aldehydes, Ketones (220–180)	Acids and Derivatives (180–160)	Aromatic Hydrocarbons (160–105)	Pure Aromatic, No Substitution (140–125)	Alcohols, Esters, Sugars (60–105)	CH3O Group in Lignin (60–55)	Aliphatic Carbohydrates (55–1)
			DMSO				
PS, standard, $CuSO_4$	2.75	32.09	11.72	10.31	6.36	5.26	41.82
PS, standard, $NiSO_4$	1.88	24.69	17.93	4.76	15.51	5.94	34.05
PS, standard, $CuSO_4$	2.93	19.39	17.69	8.16	12.65	4.09	43.25
SS, standard, 20 min	1.05	17.19	30.51	18.13	9.34	3.93	37.98
PS, standard, Zeolite	0.00	30.80	12.62	6.96	7.55	7.80	41.23
			D_2O				
PS, standard, $CuSO_4$	2.51	11.15	20.05	7.41	3.19	8.94	54.16
PS, standard, $NiSO_4$	4.08	24.21	16.15	8.95	11.24	3.65	40.67
PS, standard, $CuSO_4$	3.89	24.14	16.47	12.11	24.57	5.34	25.59
SS, standard, 20 min	0.18	13.49	13.83	8.42	12.04	4.96	55.50
PS, standard, Zeolite	0.00	22.70	15.75	6.98	9.46	7.12	44.97

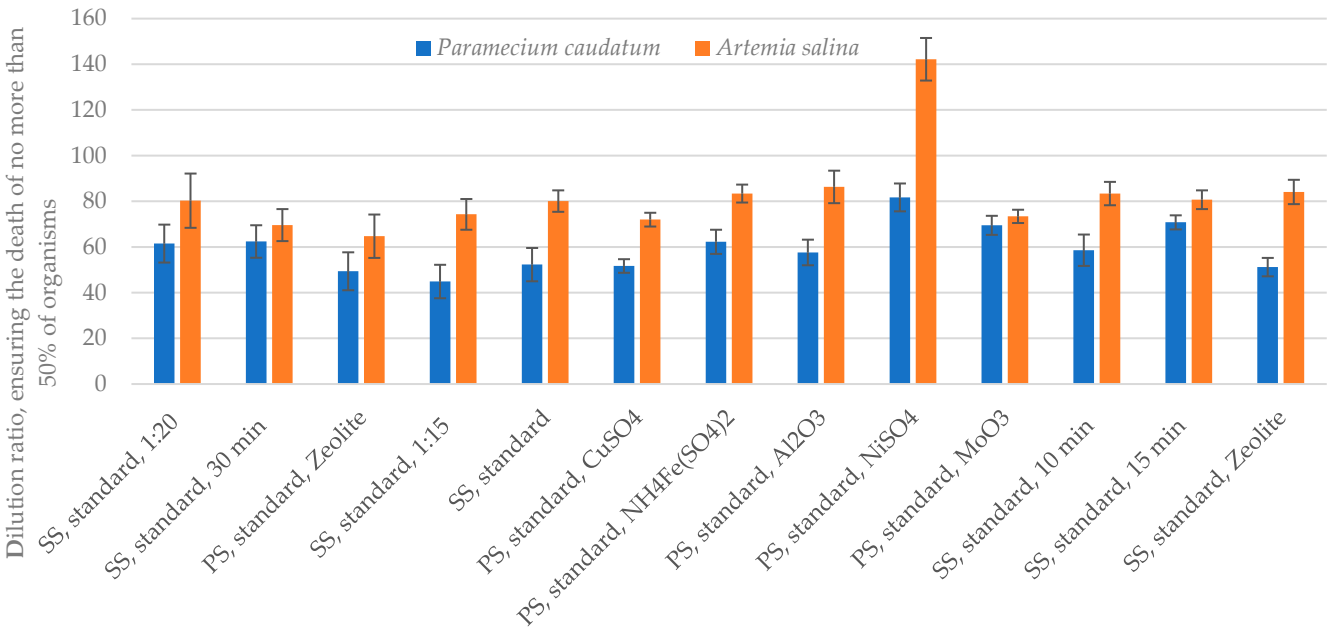


Figure 5. Dilution ratio, ensuring the death of no more than 50% of organisms of *Paramecium caudatum* and *Artemia salina*, for various HTL-AP samples. Describing the HTL condition, the following abbreviations have been adopted: SS—secondary sludge, PS—primary sludge, standard—mean standard conditions (temperature 260 °C, time 20 min, raw material to water ratio 1:10). In the case of using homogeneous and heterogeneous catalysts, the names of catalysts are given. If the process was carried out under conditions different from the standard, the variable parameter is indicated separately (for example, "1:15" means that another biomass: water ratio was taken).

A similar picture emerged when *Artemia salina* was used as a test organism: the sample containing nickel sulfate was the most toxic (DR_{50} = 142.2); the samples from the processing of sludge with the addition of zeolite (DR_{50} = 64.7) and sludge without a catalyst (DR_{50} = 69.6) were the least toxic for crustaceans.

Table 6 shows the results of determining the toxicity of aqueous samples using ciliates *Paramecium caudatum ciliates* and *Artemia salina* crustaceans as test organisms, as well as the degree of dilution of HTL-AP.

Table 6. Results of biotesting of HTL-AP toxicity on *Paramecium caudatum* and *Artemia salina*.

Sample	Dilution	*Paramecium caudatum*			*Artemia salina*		
		Death Rate, %	Toxicity Level *	DR_{50}	Death Rate, %	Toxicity Level	DR_{50}
S. sludge, standard, 1:20	1:1	100 ± 0	HT	61.5 ± 8.3	100 ± 1	HT	80.3 ± 11.9
	1:10	66 ± 8	HT		71 ± 4	HT	
	1:100	30 ± 6	LT		42 ± 8	LT	
	1:1000	0	NT		0	NT	
S. sludge, standard, 30 min	1:1	100 ± 1	HT	62.4 ± 7.1	0	HT	69.6 ± 7.0
	1:10	65 ± 7	HT		75 ± 7	HT	
	1:100	26 ± 3	LT		33 ± 6	LT	
	1:1000	0	NT		0	NT	
P. sludge, standard, Zeolite 2 g	1:1	100 ± 2	HT	49.4 ± 8.3	100 ± 3	HT	64.7 ± 9.5
	1:10	56 ± 8	HT		70 ± 9	HT	
	1:100	15 ± 3	LT		30 ± 6	LT	
	1:1000	0	NT		0	NT	
S. sludge, standard, 1:15	1:1	100 ± 1	HT	44.9 ± 7.3	100 ± 2	HT	74.3 ± 6.7
	1:10	63 ± 9	HT		62 ± 7	HT	
	1:100	14 ± 2	LT		40 ± 3	LT	
	1:1000	0	NT		0	NT	
S. sludge, standard, 20 min	1:1	100 ± 0	HT	52.3 ± 7.3	100 ± 0	HT	80.1 ± 4.7
	1:10	62 ± 10	HT		80 ± 5	HT	
	1:100	20 ± 4	LT		42 ± 3	LT	
	1:1000	0	NT		0	NT	
P. sludge, standard, $CuSO_4$ 2 g	1:1	100 ± 1	HT	51.7 ± 3.0	100 ± 2	HT	72.0 ± 3.0
	1:10	60 ± 3	HT		75 ± 4	HT	
	1:100	22 ± 2	LT		35 ± 1	LT	
	1:1000	0	NT		0	NT	
P. sludge, standard, $NH_4Fe(SO_4)_2 \cdot 12H_2O$ 2 g	1:1	100 ± 0	HT	62.3 ± 5.3	100 ± 1	HT	83.4 ± 3.9
	1:10	55 ± 5	HT		87 ± 5	HT	
	1:100	33 ± 2	LT		42 ± 2	LT	
	1:1000	0	NT		0	NT	
P. sludge, standard, Al_2O_3 2 g	1:1	100 ± 1	HT	57.6 ± 5.6	100 ± 2	HT	86.3 ± 7.1
	1:10	71 ± 6	HT		85 ± 6	HT	
	1:100	22 ± 2	LT		43 ± 3	LT	
	1:1000	0	NT		0	NT	
P. sludge, standard, $NiSO_4$ 2 g	1:1	100 ± 0	HT	81.7 ± 6.1	100 ± 1	HT	142.2 ± 9.3
	1:10	77 ± 5	HT		80 ± 6	HT	
	1:100	45 ± 3	LT		63 ± 2	HT	
	1:1000	0	NT		8 ± 1	NT	
P. sludge, standard, MoO_3 2 g	1:1	100 ± 2	HT	69.5 ± 4.2	100 ± 1	HT	73.4 ± 2.9
	1:10	73 ± 6	HT		87 ± 3	HT	
	1:100	46 ± 2	LT		33 ± 1	LT	
	1:1000	0	NT		0	NT	
S. sludge, standard, 10 min	1:1	100 ± 1	HT	58.6 ± 6.9	100 ± 1	HT	83.4 ± 5.1
	1:10	62 ± 7	HT		83 ± 7	HT	
	1:100	29 ± 4	LT		42 ± 2	LT	
	1:1000	0	NT		0	NT	
S. sludge, standard, 15 min	1:1	100 ± 0	HT	70.8 ± 3.1	100 ± 0	HT	80.7 ± 4.1
	1:10	59 ± 3	HT		67 ± 3	HT	
	1:100	39 ± 2	LT		42 ± 2	LT	
	1:1000	0	NT		0	NT	
S. sludge, standard, Zeolite 2 g	1:1	100 ± 1	HT	51.2 ± 4.0	100 ± 2	HT	84.1 ± 5.3
	1:10	61 ± 2	HT		75 ± 6	HT	
	1:100	24 ± 2	LT		43 ± 2	LT	
	1:1000	0	NT		0	NT	

Note: * NT—non-toxic, LT—low-toxic, HT—highly-toxic.

Based on biotesting results, it was determined that all wastewater samples were toxic to *Paramecium caudatum*. A toxic effect on ciliates was also observed after tenfold dilution with dechlorinated water, which resulted in the death of more than half of the *Paramecium* in all studied samples. Water samples from the HTL of primary sludge with the use of $NiSO_4$ of catalysts were the most toxic, killing 77% even if diluted tenfold. The samples

from the primary sludge HTL with iron-ammonium alum as a catalyst were the least toxic, killing 55% of the *Paramecia*. At 100-fold dilution, a death rate was observed of 12% to 46% of ciliates compared to the control sample. The 100-fold dilution of the source water had a mildly toxic effect on ciliates. A 1000-fold dilution did not have a toxic effect on the selected test organisms.

According to the results of biotesting with crustaceans as test organisms, all samples of HTL-AP had a toxic effect on the test organisms, resulting in the death of 100% of the organisms. A 10-fold dilution was also associated with a toxic effect on nauplii and an increase in mortality from 61.6% (HTL-AP from secondary sludge processing) to 86.6% (HTL-AP from primary sludge processing using Al_2O_3 as a catalyst). A slightly toxic effect on test organisms was observed with a 100-fold dilution, resulting in mortality rates ranging from 30% to 43.3%. Even when diluted 100 times, HTL-AP from primary sludge processing using MoO_3 as a catalyst was toxic to crustacean nauplii, with an average mortality of 63.3%. A 1000-fold dilution did not have a toxic effect on the test organisms.

Thus, it is worth noting that the initial wastewater has a toxic effect on both test organisms and leads to 100% death of both ciliates and crustaceans. Tenfold dilution also has a toxic effect on sludge hydrobionts. Dilution by 100 times exhibits slightly toxic properties and has a detrimental effect on an average of 29% of ciliates and 45% of crustaceans compared to the control sample. A 1000-fold dilution forms samples that do not have a toxic effect on the test organisms.

Furthermore, using a graphical method, the dilution ratio, ensuring the death of no more than 50% of organisms (DR_{50}) was determined in order to identify the most toxic wastewater sample, resulting in the death of 50% of the test organisms under study. Table 6 also includes the DR_{50} values for *Parameciun caudatum* and *Artemia salina*.

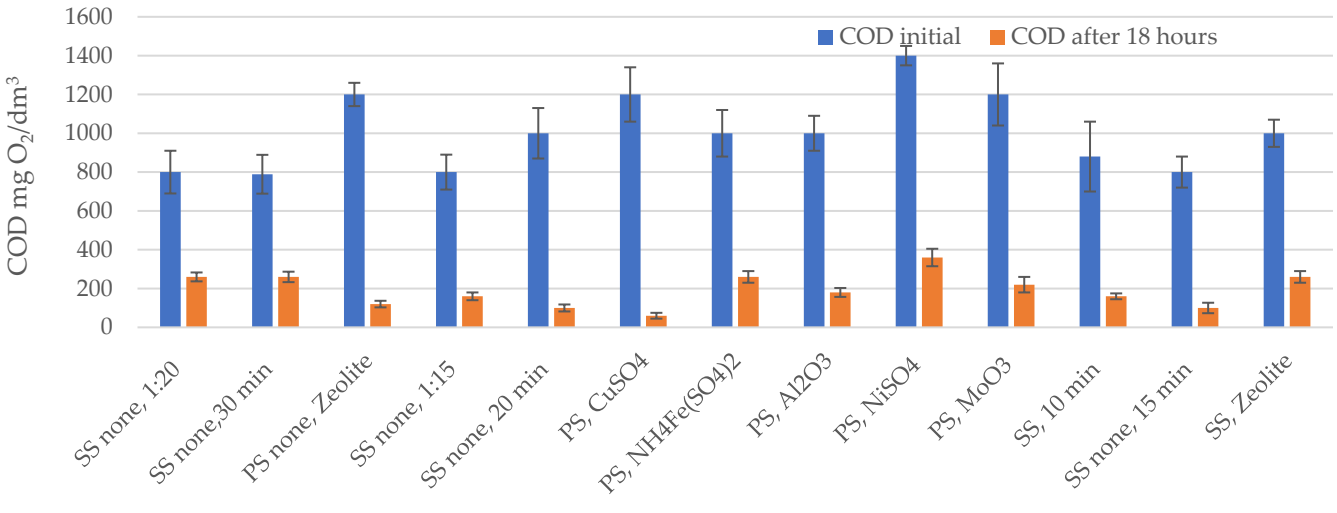
According to the toxicity results obtained using the *Paramecium caudatum* test organisms, the most toxic sample in terms of DR_{50} is HTL-AR from primary sludge processing using MoO_3 as a catalyst (DR_{50} 81.7), and the least toxic for ciliates is HTL-AR from primary sludge processing using Al_2O_3 as a catalyst (DR_{50} 44.9).

The most toxic sample in terms of DR_{50}, according to the experimental results on toxicity using the *Artemia salina* test organisms, was the HTL-AR sample from primary sludge processing using MoO_3 as a catalyst (DR_{50} 142.2); the HTL-AR sample from primary sludge processing using Al_2O_3 as a catalyst also showed fewer toxic properties for crustaceans (DR_{50} 61.2).

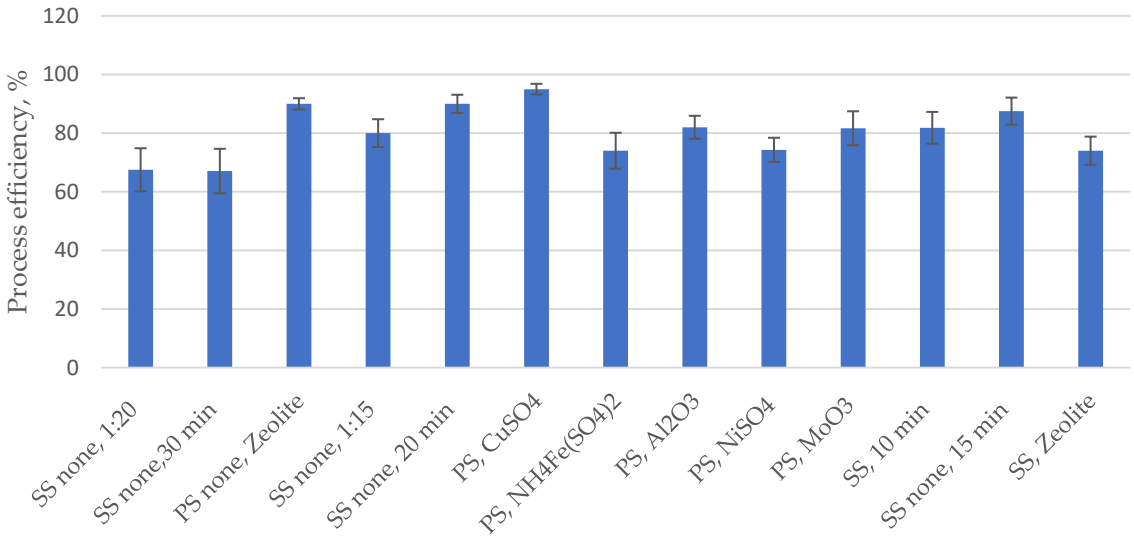
3.3. The Results of the Aerobic Biological Treatment of HTL-AP

The HTL-AP was diluted 10 times, since the presence of amides and other heterocyclic nitrogen-containing compounds have the properties of surfactants. When aerating wastewater with a high concentration of surfactants, strong foam is formed, resulting in the flotation and removal of sludge microorganism cells with foam bubbles.

After aerating the sludge and aqueous phase suspension for 18 h, the wastewater was filtered through the paper filter, and the COD value was determined. The results of cultivating activated sludge from the wastewater treatment plants of petrochemical enterprises are shown in Figure 6. The COD value for most samples at the end of the purification time was in the range of 100–200 mgO/dm^3, with the exception of the HTL-AP sample using nickel sulfate as a catalyst (COD 382 mgO/dm^3), but it should be noted that the culture efficiency for this sample, as for most samples, was 74%. The high post-cultivation COD values for this HTL-AP sample are likely due to the initially high organic content in the original sample. Cultivation efficiency in terms of COD in average was 70%. However, for HTL-AP samples obtained by processing secondary sludge without catalysts and primary sludge using copper sulfate and zeolite as catalysts, the efficiency of organic compounds assimilation by activated sludge was 90%.



(**a**)



(**b**)

Figure 6. Efficiency of aerobic biological purification of HTL-AP: (**a**) change in COD in the process of cultivation of activated sludge; (**b**) efficiency of the process, %.

The components of HTL-AP of secondary and primary sludge can be biologically oxidized and can be subjected to biological degradation by activated sludge microorganisms, according to the experimental results. It is necessary to pre-treat water, remove toxic substances (metals, surfactants), or isolate specialized cultures of bacteria from activated sludge that are resistant to toxic substances of the aqueous phase in order to increase the effectiveness of removing organic substances.

4. Conclusions

According to the research results, the aqueous phase formed during the hydrothermal liquefaction of primary and secondary sludge has a high level of organic pollution (TOC = 4.171–9.55 g/dm^3, COD = 7.89–14.0 g/dm^3, BOD_5 = 5.955–8.137 g/dm^3) and high biological toxicity for traditional test organisms (DR_{50} varied within 64.7–142.2 and 44.9–81.7 for *Artemia salina* and *Paramecium caudatum*, respectively). It is impossible to apply the biological treatment for HTL-AP without dilution or pre-treatment. Aerobic

biological treatment of HTL-AP with sludge from an industrial waste water plant (from a petrochemical plant), allowed the mineralization of 67–95% of organic substances. Further research on HTL-AP biological treatment should be aimed at isolating the most effective and stable organisms from the consortium of activated sludge organisms and selecting conditions for the preliminary physicochemical treatment of wastewater. The development of wastewater treatment systems will increase the environmental safety and commercial attractiveness of the HTL process.

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References

1. Kulikova, Y.; Sukhikh, S.; Ivanova, S.; Babich, O.; Sliusar, N. Review of Studies on Joint Recovery of Macroalgae and Marine Debris by Hydrothermal Liquefaction. *Appl. Sci.* **2022**, *12*, 569. [CrossRef]
2. Leng, L.; Li, J.; Wen, Z.; Zhou, W. Use of microalgae to recycle nutrients in aqueous phase derived from hydrothermal liquefaction process. *Bioresour. Technol.* **2018**, *256*, 529–542. [CrossRef] [PubMed]
3. Watson, J.; Wang, T.; Si, B.; Chen, W.-T.; Aierzhati, A.; Zhang, Y. Valorization of hydrothermal liquefaction aqueous phase: Pathways towards commercial viability. *Prog. Energy Combust. Sci.* **2019**, *77*, 100819. [CrossRef]
4. Leng, L.; Yang, L.; Chen, J.; Hu, Y.; Li, H.; Li, H.; Jiang, S.; Peng, H.; Yuan, X.; Huang, H. Valorization of the aqueous phase produced from wet and dry thermochemical processing biomass: A review. *J. Clean. Prod.* **2021**, *294*, 126238. [CrossRef]
5. Ekpo, U.; Ross, A.; Camargo-Valero, M.; Williams, P. A comparison of product yields and inorganic content in process streams following thermal hydrolysis and hydrothermal processing of microalgae, manure and digestate. *Bioresour. Technol.* **2016**, *200*, 951–960. [CrossRef]
6. Leng, L.; Zhou, W. Chemical compositions and wastewater properties of aqueous phase (wastewater) produced from the hydrothermal treatment of wet biomass: A review. *Energy Sources Part A Recover. Util. Environ. Eff.* **2018**, *40*, 2648–2659. [CrossRef]
7. Biller, P.; Ross, A.; Skill, S.; Lea-Langton, A.; Balasundaram, B.; Hall, C.; Riley, R.; Llewellyn, C. Nutrient recycling of aqueous phase for microalgae cultivation from the hydrothermal liquefaction process. *Algal Res.* **2012**, *1*, 70–76. [CrossRef]
8. Boer, D.F.; Valette, J.; Commandre, J.M.; Fournier, M.; Thevenon, M.F. Slow pyrolysis of sugarcane bagasse for the production of char and thepotential of its by-product for Wood Protection. *J. Renew. Mater.* **2021**, *9*, 97–117. [CrossRef]
9. Van Aken, P.; Broeck, R.V.D.; Degrève, J.; Dewil, R. The effect of ozonation on the toxicity and biodegradability of 2,4-dichlorophenol-containing wastewater. *Chem. Eng. J.* **2015**, *280*, 728–736. [CrossRef]
10. Jayakody, L.N.; Johnson, C.W.; Whitham, J.M.; Giannone, R.J.; Black, B.A.; Cleveland, N.S.; Klingeman, D.M.; Michener, W.E.; Olstad, J.L.; Vardon, D.R.; et al. Thermochemical wastewater valorization via enhanced microbial toxicity tolerance. *Energy Environ. Sci.* **2018**, *11*, 1625–1638. [CrossRef]
11. Reza, M.T.; Freitas, A.; Yang, X.; Coronella, C.J. Wet Air Oxidation of Hydrothermal Carbonization (HTC) Process Liquid. *ACS Sustain. Chem. Eng.* **2016**, *4*, 3250–3254. [CrossRef]
12. Thomsen, L.B.S.; Anastasakis, K.; Biller, P. Wet oxidation of aqueous phase from hydrothermal liquefaction of sewage sludge. *Water Res.* **2021**, *209*, 117863. [CrossRef] [PubMed]
13. Li, R.; Liu, D.; Zhang, Y.; Zhou, J.; Tsang, Y.F.; Liu, Z.; Duan, N.; Zhang, Y. Improved methane production and energy recovery of post-hydrothermal liquefaction waste water via integration of zeolite adsorption and anaerobic digestion. *Sci. Total. Environ.* **2018**, *651*, 61–69. [CrossRef] [PubMed]
14. Zhou, Y.; Schideman, L.; Zheng, M.; Martin-Ryals, A.; Li, P.; Tommaso, G.; Zhang, Y. Anaerobic digestion of post-hydrothermal liquefaction wastewater for improved energy efficiency of hydrothermal bioenergy processes. *Water Sci. Technol.* **2015**, *72*, 2139–2147. [CrossRef] [PubMed]
15. Sabio, E.; González, E.; González, J.; González-García, C.; Ramiro, A.; Gañan, J. Thermal regeneration of activated carbon saturated with p-nitrophenol. *Carbon* **2004**, *42*, 2285–2293. [CrossRef]
16. He, Y.; Li, X.; Xue, X.; Swita, M.S.; Schmidt, A.J.; Yang, B. Biological conversion of the aqueous wastes from hydrothermal liquefaction of algae and pine wood by Rhodococci. *Bioresour. Technol.* **2017**, *224*, 457–464. [CrossRef]
17. Posmanik, R.; Labatut, R.A.; Kim, A.H.; Usack, J.G.; Tester, J.W.; Angenent, L.T. Coupling hydrothermal liquefaction and anaerobic digestion for energy valorization from model biomass feedstocks. *Bioresour. Technol.* **2017**, *233*, 134–143. [CrossRef]

18. Tommaso, G.; Chen, W.-T.; Li, P.; Schideman, L.; Zhang, Y. Chemical characterization and anaerobic biodegradability of hydrothermal liquefaction aqueous products from mixed-culture wastewater algae. *Bioresour. Technol.* **2015**, *178*, 139–146. [CrossRef]
19. Gai, C.; Zhang, Y.; Chen, W.-T.; Zhou, Y.; Schideman, L.; Zhang, P.; Tommaso, G.; Kuo, C.-T.; Dong, Y. Characterization of aqueous phase from the hydrothermal liquefaction of Chlorella pyrenoidosa. *Bioresour. Technol.* **2015**, *184*, 328–335. [CrossRef]
20. Fernandez, S.; Srinivas, K.; Schmidt, A.J.; Swita, M.S.; Ahring, B.K. Anaerobic digestion of organic fraction from hydrothermal liquefied algae wastewater byproduct. *Bioresour. Technol.* **2018**, *247*, 250–258. [CrossRef]
21. Zheng, M.; Schideman, L.C.; Tommaso, G.; Chen, W.-T.; Zhou, Y.; Nair, K.; Qian, W.; Zhang, Y.; Wang, K. Anaerobic digestion of wastewater generated from the hydrothermal liquefaction of Spirulina: Toxicity assessment and minimization. *Energy Convers. Manag.* **2017**, *141*, 420–428. [CrossRef]
22. Mohanty, S.; Patel, P.; Jha, E.; Panda, P.K.; Kumari, P.; Singh, S.; Sinha, A.; Saha, A.K.; Kaushik, N.K.; Raina, V.; et al. In vivo intrinsic atomic interaction infer molecular eco-toxicity of industrial TiO_2 nanoparticles via oxidative stress channelized steatosis and apoptosis in Paramecium caudatum. *Ecotoxicol. Environ. Saf.* **2022**, *241*, 113708. [CrossRef] [PubMed]
23. Amutha, V.; Aiswarya, D.; Deepak, P.; Selvaraj, R.; Tamilselvan, C.; Perumal, P.; Balasubramani, G. Toxicity potential evaluation of ethyl acetate extract of Cymodocea serrulata against the mosquito vectors vis-a-vis zebrafish embryos and Artemia salina cysts. *S. Afr. J. Bot.* **2023**, *152*, 230–239. [CrossRef]
24. Standard Methods for the Examination of Water and Wastewater. 5220 Chemical Oxygen Demand (Cod). Available online: https://www.standardmethods.org/doi/full/10.2105/SMWW.2882.103?role=tab (accessed on 5 April 2023).
25. ISO 5815-1:2019(en) Water Quality—Determination of Biochemical Oxygen Demand after n Days (BODn)—Part 1: Dilution and Seeding Method with Allylthiourea Addition. Available online: https://dgn.isolutions.iso.org/obp/ui#!iso:std:iso:5815:-1:ed-2:v1:en (accessed on 5 April 2023).
26. Kulikova, Y.; Krasnovskikh, M.; Sliusar, N.; Orlov, N.; Babich, O. Analysis and Comparison of Bio-Oils Obtained by Hydrothermal Liquefaction of Organic Waste. *Sustainability* **2023**, *15*, 980. [CrossRef]
27. Zhao, J.; Wang, M.; Saroja, S.G.; Khan, I.A. NMR technique and methodology in botanical health product analysis and quality control. *J. Pharm. BioMed. Anal.* **2021**, *207*, 114376. [CrossRef]
28. Saito, T.; Nakaie, S.; Kinoshita, M.; Ihara, T.; Kinugasa, S.; Nomura, A.; Maeda, T. Practical guide for accurate quantitative solution state NMR analysis. *Metrologia* **2004**, *41*, 213–218. [CrossRef]
29. Kryazhevskikh, A.A.; Bardina, V.I.; Sklyarova, N.A. Biotesting methods for the detection of drugs in the aquatic environment. *Pharm. Formulas* **2022**, *4*, 61–69. [CrossRef]
30. Rajabi, S.; Ramazani, A.; Hamidi, M.; Naji, T. Artemia salina as a model organism in toxicity assessment of nanoparticles. *DARU J. Pharm. Sci.* **2015**, *23*, 20. [CrossRef]
31. Galitskaya, P.Y.; Selivanovskaya, S.Y.; Gumerova, R.K. *Testing of Wastes, Soils, Materials Using Living Systems: Tutorial-Method Allowance*; Kazan University Publishing House: Kazan, Russia, 2014; pp. 14–23. (In Russian)
32. Basar, I.A.; Liu, H.; Carrere, H.; Trably, E.; Eskicioglu, C. A review on key design and operational parameters to optimize and develop hydrothermal liquefaction of biomass for biorefinery applications. *Green Chem.* **2021**, *23*, 1404–1446. [CrossRef]
33. Khalilova, A.A.; Yakovlev, A.V.; Sirotkin, A.S. *Comparative Assessment of the Toxicity of Wastewater Containing Chromium and Nickel Ions Using Various Test Organisms*; Bulletin of the Kazan Technological University: Kazan, Russia, 2010; Volume 10, pp. 392–400. (In Russian)