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Ecotoxicity of Purified Industrial Waste Water

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Abstract Purified industrial waste water (PIWW) has been evaluated for probable toxicity using test-systems with *Pseudorasbora parva* (topmouth gudgeon) and *Lepidium sativum* L (garden cress). The acute toxicity of PIWW was calculated according to mortality of *Pseudorasbora parva* in dilutions 1, 5, 10, 25, 50, 100 and 200 times, and distillate water (DW) was used as a control. The LC_{50} has been calculated after 96 h of treatment and it was at approximately 8x (7.69) times dilution of PIWW. The toxic effect of PIWW with and without copper ions, added as $CuSO_4$ have been measured using *Lepidium sativum* L. The comparison of the toxic effects of the same concentrations of copper in PIWW and DW, mixed and non-mixed contamination has been evaluated. It was found that Cu^{+2} has inhibitory effects on the root's and stem's growth of *Lepidium sativum* L seeds, and that effect appears in concentrations over 2 mg/l Cu^{+2}. The 50% inhibition of root's growth in DW was EC_{50}=7.26 mg/l of copper ions, while for PIWW that concentration was EC_{50} =17.23 mg/l Cu^{+2}. The calculated EC_{50} for stem's growing in DW was 54.57 mg/l Cu^{+2} and 72.07 mg/l Cu^{+2} in PIWW. The observed EC_{50} differences in DW and PIWW perhaps are due to the formation of ligand compounds among copper cations and other impurities in the waste water and hence as consequences the reducing of free Cu^{+2} or their bioavailability, hereafter reduce copper toxicity. It was registered that PIWW diminished growth inhibitory effect of copper ions on *Lepidium sativum* L seeds lessening its amount by involving free Cu^{+2} in complexes with other waste products.

Keywords LC_{50}, EC_{50}, Copper Ions, Purified Industrial Waste Water, *Lepidium sativum* L., *Pseudorasbora parva*

1. Introduction

Heavy metals are naturally occurring elements and significant environmental pollutants; their toxicity is a crucial problem with increasing ecological, nutritional and environmental importance, especially in regions with pronounced anthropogenic pressure [40, 61, 4, 8, 15, and 19]. The Environmental Quality Standards (EQSs) for freshwater are based on laboratory experiments that are often carried out with inorganic metal salts, where the ions are more bioavailable [10]. It is well-known that the bioavailability of metals is significantly influenced by pH, alkalinity and hardness of water, and differs for each metal and its speciation [19]. The bioavailability of ions was recognized as a key factor when setting EQSs for metals [10, 19]. The differences in the bioavailability of metals in laboratory tests compared to field situation are a crucial topic when the risk levels are projected [10, 19]. Conversion of the metal ions toxicity effects to the field situation is a hard task, where metals are incorporated in complexes with dissolved organic matter (DOM) and other components of the system [10]. Even acute concentration of different toxicant differs with its speciation and with environmental conditions [45]. In the conversion of the toxicity often water pH is not taken into account; though there is clear evidence that just pH can greatly modify the toxicity of the pollutants [31]. Ordinary chronic toxicity is much less affected by hardness of water than the acute [19, 22]. For instance, in case of Cu^{+2} the water hardness did not affect chronic toxicity to *Daphnia*, but affected acute toxicity [22]. Cu^{+2} are acutely lethal to freshwater fish in soft water at low concentrations ranging from 10 – 20 ppb [41]. Oronsaye and Ogbebo (1997) reported LC_{50} of 0.4mg/l for *Clarias gariepinus* exposed to 96 hour of copper sulphate in soft water [47].

Copper is an essential trace element, naturally occurs in the aquatic environment in low concentrations and serves as a minor nutrient for plants and animals [16, 19, 66], but in higher concentrations is an actually toxic for aquatic life [16, 19, 26]. Major U.S. aquifers have Cu^{+2} concentrations less than 10 ppb [36], Canadian freshwaters 1-8 ppb Cu^{+2} [6], streams in Bristol Bay 0.04 - 5.60 ppb Cu^{+2} [67], and for seawater copper concentrations are recorded generally less than 1 ppb [42]. Copper is a toxic metal to aquatic organisms and ecosystems, and has effects on fish, invertebrates, and amphibians; which are equally sensitive to chronic toxicity [62, 32, and 57]. Copper in the lake is bioavailable and bioaccumulate by organisms up to high levels in many different organs and some effects of long-term toxicity of copper on benthic community and planktonic biomass were pointed out [12, 63]. Copper is moderately soluble in water and binds easily to sediments and organic matter [16], and does not biomagnify in food webs [16, 57]. However, there is evidence of biomagnification of copper in the food chain with possible threats to human health noticed in the case study of grossly polluted Korle Lagoon [12]. Although, mammals are not so sensitive to copper toxicity as aquatic organisms, biomagnifications play a critical role in the poisoning. Toxicity in mammals include a wide range of animals and effects, such as liver cirrhosis, kidneys and brain necrosis, gastrointestinal distress, lesions, low blood pressure and fetal mortality [6, 34, 64, 65].

Aquatic habitats are susceptible for pollution because they are ultimate receptor of industrial and urban wastewater, storm water runoff, and atmospheric deposition [21, 43]. Oyewo [48] tested some metals found in the industrial effluents on five animal species: *Cypris sp.*, *Mugil sp.*, *Tilapia sp.*, *Nerita senegalensis*, and *Clibanarius africanus* that normally inhabit the Lagos Lagoon. The values on the general order of toxicity of the test metals were: Hg, Cu, Mn, and Fe, when they were tested separately. Elevated aquatic Cu^{+2} concentrations are primarily occurring near copper mining, smelting facilities and in urbanized areas [21, 25]. Higher Cu^{+2} concentrations are observed in mine-impacted Mineral Creek Colorado, where they were approximately 410 ppb [55] and around the mine impacted Copperas Brook in Vermont where they were around 4600 ppb [8]. Sansalone et al. [56] documented Cu^{+2} concentrations of 325 ppb in urban storm water run-off, such copper concentrations are lethal to fish and aquatic life [25].

The anthropogenic contamination of water involves dissolved heavy metals, detergents, DOM, etc. Low levels of detergents increase the uptake of other pollutants [1]. For instance, the mixture of anionic detergents and Cu^{+2} causes toxic effect “more-than-additive”, while the mixture of non-anionic detergent and metal ions produces toxic effect that is probably “less-than-additive” on rainbow trout [15].

The waste water mixers frequently contain detergents with high portion of phosphorus causing eutrophication [12, 50]. It has been reported that the presence of 1 mg/l detergents in water causes plankton perishes; 3 mg/l - mass *Daphne* death; 5 mg/l - fish damage [38]. In concentrations between 0.4 and 40 mg/l synthetic detergents are reported to be acutely toxic to fish [1]. Invertebrates, especially in their juvenile stages, are extremely sensitive to detergents in concentration below 0.1 mg/l. The detergents are very hazardous among the pollutants, particularly for the water organisms, they can form a layer of foam on the water surface with considerable thickness and to prevent oxygen exchange of the aqua ecosystems with all resulting consequences. Contaminated water containing detergents can hardly be purified by biochemical oxidation [39]. In many countries there are no legislations to control phosphates in soaps. In Canada phosphate-laden dish detergents are banned for sale from 2010; numerous USA municipalities banned the phosphate both in detergents and fertilizers in an effort to minimize the runoff [50].

The aim of this study is to evaluate the toxic effect of PIWW with and without higher copper concentration; and to compare the effects of the same concentrations of copper in purified waste water and distilled water.

2. Materials and Methods

2.1. Sofia Med Company

SOFIA MED S.A. is a manufacturer of a wide range of rolled and extruded products made of copper, copper alloy and zinc, such as sheets, strips, plates, discs, bars, rods, profiles, wires, which find various applications. The Sofia Med S.A. is a part of Halcor Group that manufactures metal products of copper and brass tubes, copper, zinc and brass rolled and extruded products, as well as cables. Halcor Group consists of 13 companies in 6 countries (Greece, Bulgaria, Romania, United Kingdom, France and Germany) and runs 10 plants in Greece, Bulgaria and Romania.

The Sofia Med S.A. is located in the eastern part of Sofia, Bulgaria (www.sofiamed.bg/bg). The company is engaged in the manufacture of copper, brass and titan zinc rolled products as well as copper extruded products. The company produced sheets, strips, plates, discs, bars, rods, profiles, copper and brass pipes, power and telecommunication cables, wires, etc., made of copper, copper alloy and zinc. The plant has three production workshops: foundry, rolling and extrusion workshops.

The purified industrial waste water (PIWW) of Sofia Med Company contained unsolved substances, sulphate ions, petroleum products, copper, lead, zinc, detergents and dissolved organic matter (DOM). The PIWW of the company contained soap that is used to remove oil entering during the production process. The maximum allowable toxicant concentrations (MATC) in waste water of Sofia Med Company are represented in table 1.

Table 1. Emission norms of waste water pollutant from Sofia Med Company Purification Station

Parameters	Maximum allowable toxicant concentrations (MATC) – mg/l
Active Reaction (pH)	6.5-9.0
Unsolved substances, mg/l	400
Sulphate ions, mg/l	400
Petroleum products, mg/l	15
Copper, mg/l	2.0
Lead, mg/l	2.0
Zinc, mg/l	5.0

Sofia Med S.A. operates in accordance with rigorous management systems for quality, environmental protection, occupational health and safety, as well as in accordance with ISO 9001, ISO 14001 and OHSAS 18001 [53]. The company uses private water-supply and drinking water. A safety level of Cu^{2+} in drinking water is accepted to be less than 2.0 mg/l, according to EU and government legislation [24]. The purified industrial waste water (PIWW) from the company is discharged into the Iskar River.

2.2. Description of the Conducted Tests for Evaluation of Purified Waste Water Toxicity

2.2.1. Acute Toxicity of Purified Waste Water - Biotest with *Pseudorasbora parva* (Topmouth Gudgeon)

The acute toxicity of purified waste water was tested with Pseudorasbora parva. The conditions were kept optimal for the development of fish according to ISO 7346 [39]. In the conducted experiment the temperature was constant 24℃; O_2 = 8-9 mg/l; pH varied in the samples (table 3) with different dilution of PIWW, and different toxicant concentrations (table 1).

Dilutions of PIWW used in the experiments: original PIWW = 1x, 5x, 10x, 25x, 50x, 100x and 200x times, and distilled water (DW) as a control, in total 8 variants of testing. In 8 test boxes out of 2l, survival of 7 fishes for every variant of testing was observed after 96 hours. The results are presented as a percentage of mortality with reference to the control and as probit units (PU) of the percentage of mortality in different dilutions.

The dilution of PIWW causing a 50% mortality in *Pseudorasbora parva* in 96 h was recorded.

2.2.2. Biotest with *Lepidium sativum* L (Garden Cress)

The biotest with *Lepidium sativum* L. was used to assess water toxicity. The tests were carried out on a solid substrate in pots, on floating hydrocracker stands and in petri dishes with soaked filter paper. The high sensitivity of plants at this early stage of development is very appropriate to assess the toxic effects (ISO 7346, [39]). The test is based on the high sensitivity of plants at this early stage of development, to the level of toxicants.

The preliminary preparation of the tests included:

A. Preparing the variants: Control distilled water (DW); DW CU^{+2} $mg.l^{-1}$; Original waste water (WW) with 2 $mg.l^{-1}$ Cu and other impurities; next tested concentrations were with DW + 5, 25, 50, 75, 125 and 250 mg/l Cu^{2+} (fig.1 a) and WW +5, 25, 50, 75, 125 and 250 mg/l Cu^{2+} (fig. 1 b).

B. Preparing the Petri dishes: 3 plates were prepared for every variant of testing. After sterilizing the petri dishes (with cotton and alcohol) and filter papers (autoclaved under dry steam for 30 minutes at 1 atm and 121℃), the double-layer filter paper on the base of the plate was soaked in 10 ml of DW (control) or 10 ml of corresponding solution in different variants. The filter paper on the lid of the plate was soaked in 5ml DW for the control or in solutions with different $CuSO_4$ concentrations.

C. Preparing the biotest: seeds were kept in a refrigerator for 24 hours [14], then they were soaked in the distilled water for 24 hours; 50 identical seeds were arranged at equal distances on the moistened paper on the base, and loaded plates were closed.

(a) in DW (b) in PIWW

Figure 1. The *Lepidium sativum* L. seeds after three-day exposure to different concentrations of Cu^{+2} ions: (a) in DW; (b) in PWW

The pH and temperatures of water by variants were measured using pH-meter HANNA. The laboratory tests were conducted at room temperature 18 – 20 ℃. After 96 h the length of the stems and roots were measured in *mm*. The measured indicator is sensitive to the pollution and other environmental factors that influenced seed's growth [39].

2.3. Statistical Analysis

2.3.1. Probit Analysis

Probit Analysis is commonly used in toxicology to determine the relative toxicity of chemicals to living organisms [35]. Probit analysis is a specialized regression model of binomial response (e.g. death/no death) variables [44], and the relationship between the response and the various concentrations is always sigmoid [35, 23]. Probit analysis acts as a transformation from sigmoid to linear and then runs a regression on the relationship [35, 44].

Determination of probit units (PU) and evaluation of LC_{50} can be easily done using the Finney's table (table 2), for converting % mortality to probits.

Table 2. Finney's table for converting % mortality to probit [29]

%	0	1	2	3	4	5	6	7	8	9
0	–	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.5
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
–	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

For instance, for a 17% death response, the corresponding probit would be 4.05; for a 15%, the PU would be 3.96; for 20% dead response the probit would be 4.16. Furthermore, for a 50% response (LC_{50}), the corresponding probit would be 5.00.

The results were statistically processed.

2.4. Growth Test Inhibition (GrInh %)

The growth inhibitory effect (GrInh %) of different concentrations of Cu^{+2} ions, added as $CuSO_4$ salt, has been evaluated in DW and in PWW on *Lepidium sativum* L seeds. The percentage of the mean for every variant values with reference to the control (100%) was calculated. The growth inhibition (GrInh %) of used concentrations was calculated by the formula:

$$Gr\ Inh\ \% = \frac{Gr\ control - Gr\ sample}{Gr\ control} . 100$$

EC_{50} of growth inhibition (GrInh %) for different variants were evaluated.

One-way ANOVA was performed using Statistic 7.0 software (Stat soft; www.statsoft.com) to compare the means of different treatments in each of the tests. The significance level was set at 0.05.

All results given in % were graphically represented and EC_{50} approximate value was calculated [3].

3. Results

3.1. Toxicity Identification Evaluations (TIEs) with *Pseudoraspora parva*

Acute ecotoxicological test examinations are the first step to detect the total toxic effects caused by toxicant, and virtually every hydrobiont is suitable for conducting the tests [38]. The assessment of the environmental risk values helped to determine whether the toxicant is biologically active at test doses and to define LC_{50}, which proved to be lethal causing death to 50% of the tested organisms [44].

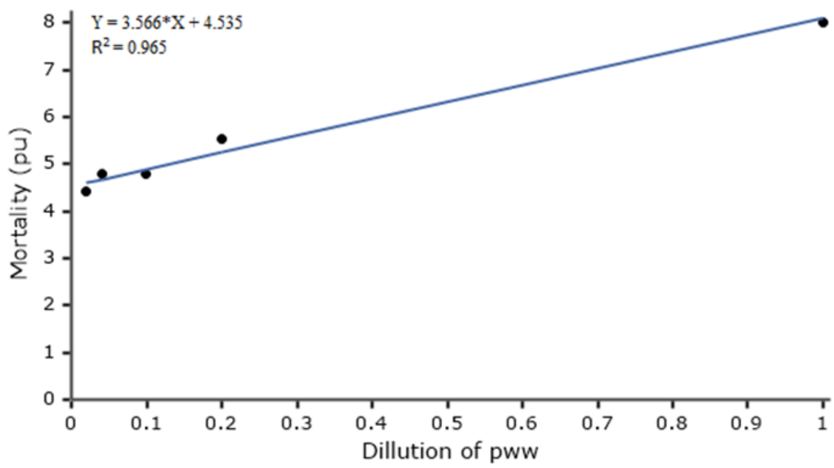
The toxic effects of purified industrial waste water (PIWW) have been evaluated on the topmouth gudgeon (*Pseudoraspora parva*) as a % of mortality after 96 h exposure to different dilutions of PIWW (table 3).

Table 3. Toxic effects of purified waste water on *Pseudoraspora parva*

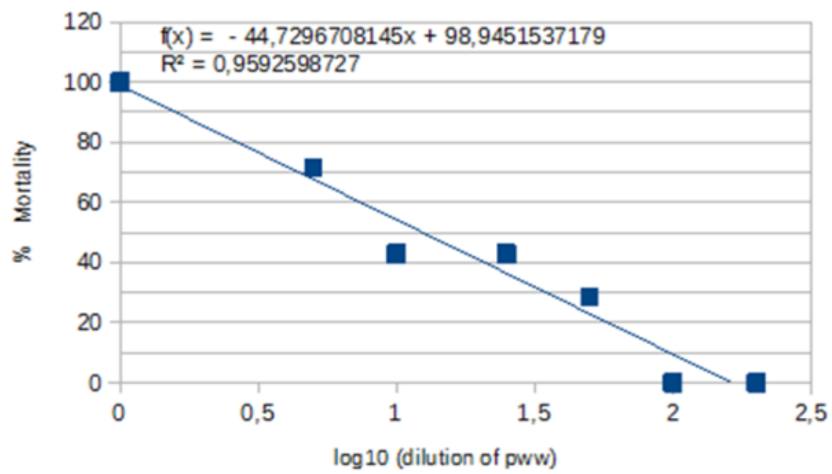
Dilution of PWW	pH (t=24℃)	Mortality (n)	Mortality %	PU
Control - DW	6,74	0	0.00	0.00
200x	6,74	0	0.00	0.00
100x	6,84	0	0.00	0.00
50x	6,97	2	28.57	4.42
25x	7,19	3	42.86	4.82
10x	7,42	3	42.86	4.82
5x	7,58	5	71.43	5.55
1x	7,52	7	100.00	8.09

Dilutions of purified waste water: 1x, 5x, 10x, 25x, 50x, 100x and 200x times or in opposite order 0.005, 0.01, 0.02, 0.04, 0.1, 0.2 and 1 (undiluted PIWW); distillated water (DW) was used as control. The temperature was 24℃, kept constant during the experiment. In undiluted PIWW after 96 h was registered 100% mortality, all fishes were died. The equation that maximum fitted to the obtained results was logarithmic f(x) = -19.4258492121 ln(x) + 98.945 with correlation coefficient $R^2 = 0.959$. 50% of mortality, LC_{50}, was detected in a range between 5 and 10 times dilutions of PIWW, and in 0.2 and 0.1 dilutions of PIWW with 71.43% and 42.86% mortality, respectively.

The probit-log concentration graph is considered as an excellent way for calculation of LC_{50} and toxicity result presentation [44]. The percentage of mortality was converted into Probit Units (PU) using the Finney's table (table 2). The results are presented in table 3 and fig.2. The concentration leading to 50% mortality after 96 h, (LC_{50}), was calculated using the equation y = 3.566*x + 4.535 of linear regression (fig.2 a).



(a) Toxic effect - PU depending of PIWW dilution
(Residual Variance = 0.002; F (Fisher) = 83.057; p < 0.0028)



(b) Toxic effect as % mortality of *Pseudoraspora parva*

Figure 2. Toxic effect of purified waste water on *Pseudoraspora parva*

The regression correlated well with the observed data with coefficient R^2=0.965. The Equation Y = b1*X + b0, has b0 = 4.535 ± 0.381 with C.I. (95%) and for b1 (slope) = 3.566 ± 1.245 with C.I. (95%). The above equation showed calculated LC_{50} to occur at 0.13 PIWW dilutions, approximately 8x (7.69) times purified waste water dilution lead to 50% mortality of *Pseudoraspora parva*.

The calculated LC_{50} based on the linear regression of mortality (%) to logarithmic dilution of PIWW (Fig.3b) obtained result 8x (7.82) times dilution of PIWW for 50% mortality of *Pseudoraspora parva*, with R^2=0.959.

Therefore, LC_{50} toxic effect appears in dilution of PIWW less than10 times.

3.2. Biotests with *Lepidium Sativum* L

Lepidium sativum L (garden cress) is a sensitive plant used as a toxicity tests object, because of its rapid growth, low cost and easy analyses [51]. The Cu^{+2} growth inhibitory effect (GrInh %) has been assessed in DW and PIWW on *Lepidium sativum* L seeds. The obtained results are represented in Table 4 and Fig.3.

Table 4. *Lepidium sativum L* growth inhibition

Growth inhibition of roots (%)					
Cu^{2+} (mg/l)	DW		PWW		Gr Inh % (PIWW-DW)
	Root's length,(mm) mean ±C.I.	Gr Inh %	Root's length, mean ±C.I. (mm)	Gr Inh%	
DW + 0	65.89 ± 3.26				
2	58.02 ± 3.64	11.94	74.91 ± 5.21	-13.70	-25.64
10	19.87 ± 2.41	69.84	44.7 ± 2.62	32.16	-37.68
25	10.56 ± 2.14	83.97	20.3 ± 2.75	69.19	-14.78
50	4.69 ± 1.29	92.88	11.53 ± 8.02	82.50	-10.38
75	3.03 ± 0.96	95.4	4.66 ± 1.08	92.93	-2.47
125	2.54 ± 0.75	96.14	3.41 ± 0.64	94.82	-1.32
250	1.83 ± 0.26	97.22	2.07 ± 0.27	96.86	-0.36
Growth inhibition of stems (%)					
Cu^{2+} (mg/l)	DW		PWW		
	Stem's length, (mm) mean ±C.I	Gr Inh %	Stem's length, (mm) mean ±SD	Gr Inh %	Gr Inh % (PIWW–DW)
DW + 0	23.29 ± 1.12				
2	20.17 ± 0.74	13.37	28.48 ± 0.54	-22.29	-35.66
10	22.71 ± 1.22	2.48	21.16 ± 0.79	9,13	6.65
25	17.09 ± 1.26	26.59	24.09 ± 1.23	-3,466	-30.056
50	12.23 ± 0.66	47.48	21.16 ± 1.05	9,13	-38.35
75	9.02 ± 1.24	61.26	10.38 ± 1.38	55,42	-5.84
125	9.48 ± 1.1	59.29	10.49 ± 0.82	54,94	-4.35
250	4.93 ± 0.48	78.81	4.15 ± 0.34	82,16	3.35

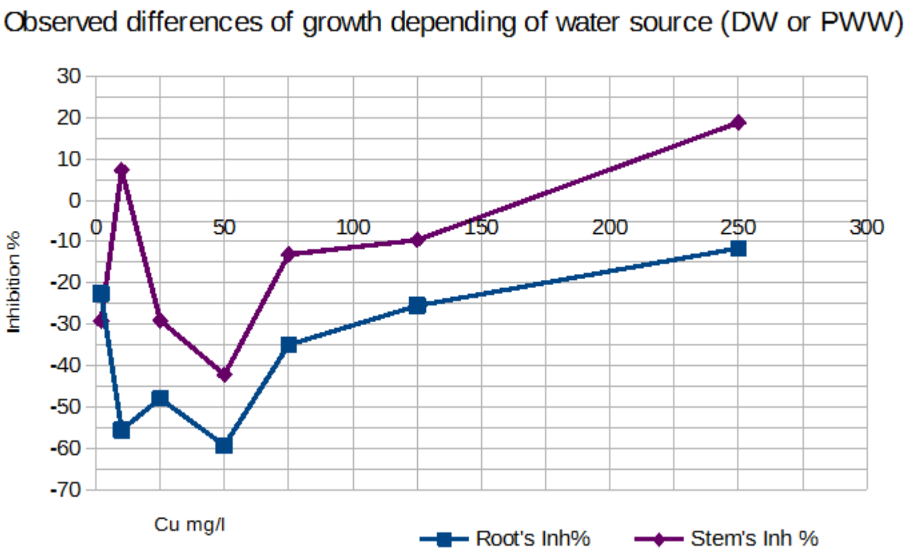


Figure 3. Reduced inhibitory effect of Cu^{+2} in PWW (Gr Inh $_{PWW}$ - Gr Inh $_{DW}$) %

It has been found that Cu^{+2} have inhibitory effect on the root's and stem's growth of *Lepidium sativum* L seeds, and it appears in concentration over 2 mg/l (Table 4; Fig.4 and 5). Also the combination of Cu^{+2} and PIWW led to reduced toxicity, and respectively, decreased inhibitory effect of the copper ions (Table 4, Fig. 3), that resulted in stimulation of root's and stem's growth.

Figures 4 and 5 represent the results of root's and stem's growth inhibition (%) in presence on different concentrations of Cu^{+2} (mg/l) in DW and PIWW. *L. sativum* L root's development was affected by Cu^{2+} metal ions in all concentrations in DW, while in pure PIWW they had a stimulatory effect on the growth of roots and stems (Fig.4 and 5).

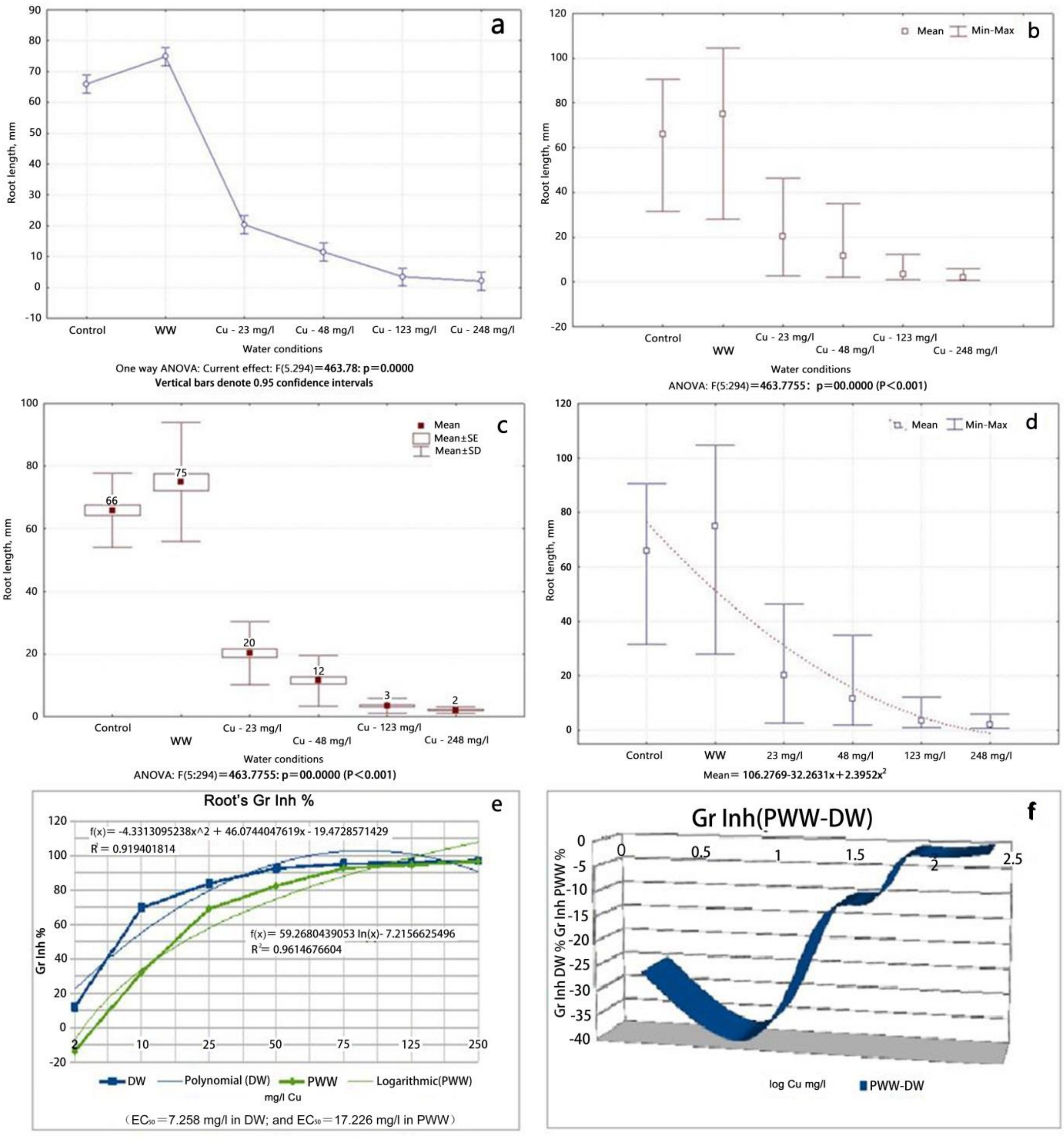


Figure 4. Root's length of *Lepidium sativum L*, Gr Inh % in dependence of Cu^{2+} (mg/l) concentrations in DW (EC_{50}=7.26 mg/l) and in PIWW (EC_{50}=17.226 mg/l); (a) Mean of root's length – PIWW stimulated seed development; (b) Mean ± SD; (c) Mean ± SE and SD; (d) Mean=106.2769-32.2631x+2.3952x^2 – the regression is negative and polynomic; (e) Growth inhibition of roots (%) in DW and PIWW (EC_{50}=7.26 mg/l in DW; and EC_{50}=17.23 mg/l in PIWW); (f) Substraction of Cu $^{2+}$ inhibit effects on roots length (PIWW-DW)%.

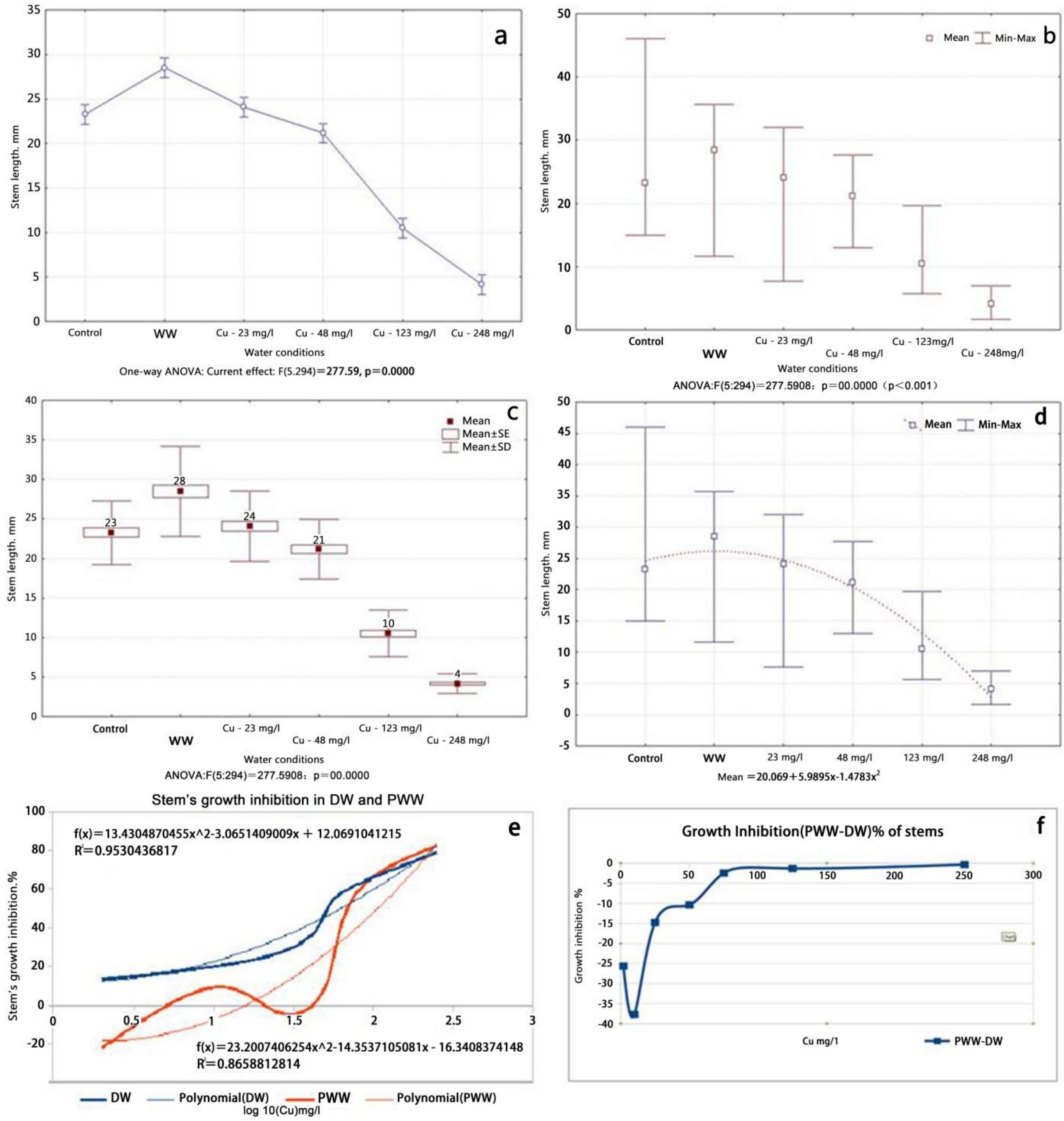


Figure 5. Inhibitory effect on *Lepidium sativum* L stem's growth by Cu $^{2+}$ in increased concentrations (mg/l) of DW (EC_{50} = 54.57 mg/l) and PIWW (EC_{50} = 72.07 mg/l); (a) Mean of variance – pure PIWW has stimulatory effect on stem's growth; (b) Mean ± SD; (c) Mean ± SE and SD; (d) Mean = 20.069+5.9895x-1.4783x2; (e) Growth inhibition of stems (%) in DW and PIWW; (f)) Substraction of Cu $^{2+}$ inhibit effects on stem's length (PIWW-DW)%.

The root's growth was inhibited by Cu^{2+} ions in the medium, the trend was polynomic with correlation coefficient R^2=0.91 in DW, and logarithmic in PIWW with R^2=0.96. 50% inhibition of root's growth appeared in two different ranges for the both water samples, in DW EC_{50} = 7.26 mg/l of copper ions, while in PIWW that concentration was EC_{50} = 17.23 mg/l Cu^{+2}(Fig.5e). Perhaps the impurities in waste water form ligand compounds with copper cations that reduced their bioavailability, and respectively their toxicity. In field studies of ecology and chemistry of upland streams, it has been discovered that copper tends to form organic complexation [10].

The stem's growth of *L. sativum* L. was inhibited with the increasing of Cu^{2+} ions concentration in the solutions (Table 4), the trends were polynomics f(x) = 13.43x2 - 3.085x + 12.069 with R^2=0.95 in DW and f(x) = 23.2x2 – 14.36x + 16.34 in PIWW with correlation coefficient R^2=0.86. The calculated EC_{50} for DW was EC_{50} = 54.57 mg/l Cu^{+2} and in PIWW EC_{50} = 72.07 mg/l Cu^{+2} (Fig.5e). The PIWW reduced the toxicity of Cu^{+2} manifested as mitigating the inhibition of growth (Fig. 5f).

The pure PIWW that contains low concentration of copper ions (2 mg/l) stimulated the stem's growth with 22.29% (Table 4; Fig. 5a).

4. Discussion

4.1. Toxicity of PIWW - Dose-Response Curve

Acute toxicity test involves estimation of LC_{50} which is the concentration that proved to be lethal causing 50% death of the tested organisms. The calculated LC_{50} for *Pseudoraspora parva* in dependence on PIWW dilution and based on probit linear regression was 0.13 of PIWW dilution, or it is approximately 8x (7.69) times dilution of purified waste water. The obtained negative regression correlated well with the observed data with coefficient R^2=0.965 (Fig. 3a). The calculated LC_{50} based on mortality (%) of topmouth gudgeon to logarithmic PIWW dilution (Fig.3b) showed similar result at 8x (7.82) times dilution of PIWW, and carried out 50% mortality of *Pseudoraspora parva*, with R^2=0.959. Therefore, LC_{50} toxic effect appears in dilutions of PIWW less than10 times, closed to 8 times.

Probit analysis is commonly used in toxicology to determine the relative toxicity of chemicals to living organisms [44]. Whenever, a chemical substance was administered to a biological system, different types of interactions occurred leading to series of responses [2]. In most studies with the same toxicant and species, variations in toxicity values were frequently observed [44], due to the minor differences in the experiment, environmental conditions, water quality, weight, age, and gender of the fish [7, 45, 46].

Nowadays, probit analysis is still the preferred statistical method in understanding dose-response relationships in acute toxicity analyses and for evaluation of LC_{50} [35, 44].

4.2. Toxicity of Cu^{+2} ($CuSO_4$) in DW - Dose-Response Curve

The most bioavailable and therefore most toxic form of copper is the Cu^{+2} - cupric ion [4, 18, 49, 57]. The toxicity of copper to aquatic life related primarily to the activity of the cupric (Cu^{2+}) ion, and possibly to some of the hydroxy complexes [5, 17, 33, 49, 52, and 54]. Information on the relationship of metal speciation to chronic metal toxicity is lacking, but data from acute toxicity experiments indicate that cupric ion is the copper species most toxic to fish [4, 15, 18, and 49]. The copper toxicity to *Thalassiosira pseudonana* has been found that is related to cupric ion activity and not to total copper concentration [60].

The obtained dose-respond curves of roots and stems growth in dependence of increased copper concentration in DW are polynomic (Fig. 4e, 5e). There were no good simple linear regression, but based on similar results obtained by other authors, we came to the conclusion that Cu and Pb are not significant predictors in the context of the regressions. Despite the fact that they are significant stressors, there were no apparent effects seen from the simple linear regressions [10].

4.3. Mixed Toxicity - Dose-Response Curve

Usually the toxic effect is expressed as LC_{50} values for mortality and EC_{50} values for inhibition of growth [27]. Many outcomes of toxicity and ecotoxicity examinations using the same toxicant and test organism differ in LC_{50} and EC_{50} values [27, 44]. Even at low concentrations 2 mg/l Cu^{+2} introduced as $CuSO_4$ modify the growth rate of unicellular algae and their photosynthetic rates; and a better growth rate was obtained by addition of chelators to the solution [58]. The organic chelators as EDTA, humic acids, glutamic acid, waste-water and detergents decreased Cu^{+2} bioavailability and hence its toxicity [9]. The obtained dose-response curves of root's and stem's growth inhibition confirmed that PIWW minimize the negative effect of Cu^{+2} (Table 4; Fig. 4f and 5f).

Copper naturally occurs in waters as divalent cupric ion in free or complexed forms [16]. Not all species of a given metal possess the same toxicity [37]. Toxicity of copper depends on the physicochemical form of the metal, and the organic substances are able to detoxify and complex copper [30]. In unpolluted water copper exist as copper carbonate [16]. In waste water free ion concentrations are lower, because the metal is complexed with dissolved ligands, or possibly bound to colloidal particles [10, 16]. Under normal conditions, most of the copper in solution is in complexed form, as both organic and inorganic ligands complex copper [16]. Copper express great tendency to form complexes with dissolved organic matter (DOM), the tendency of the metal ions to be complexed increases in the order Zn < Cd < Ni < Pb < Cu. Nevertheless, in natural water with DOM, copper is present mostly in organically complexed forms with biotic ligand, and therefor has reduced bioavailability and toxicity effect (Fig. 6; [10, 16]).

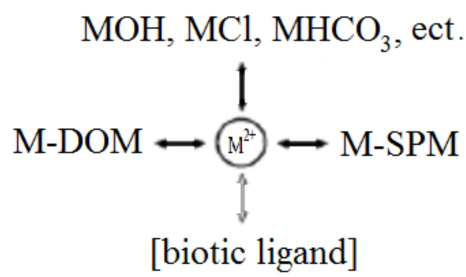


Figure 6. A schematic diagram of metal speciation, including binding to a 'biotic ligand'. DOM - dissolved organic matter, SPM - suspended particulate matter [10]

In polluted waters, complexes of copper with organic material will prevail [16]. The presence of a free cupric ion in eutrophic waters is generally low and may be less than

1%, where complexes predominate. The cupric ion is highly reactive and forms moderate to strong complexes and precipitates with many inorganic and organic constituents (carbonate, phosphate, amino acids, and humate). Most organic and inorganic copper complexes and precipitates appear to be much less toxic than free cupric ion and tend to reduce toxicity attributable to total copper concentration [4, 13, and 16]. Williams (1969) found that 5 to 25% of the total copper concentrations are associated with organic matter [68], while Batley and Florence (1976) recorded 6 to 40% of Cu^{2+} associated with DOM [11]. Several types of organic compounds can bind Cu ions and thus to reduce its toxicity [59]. This greatly complicates the interpretation and application of available toxicity data, because the proportion of free cupric ion presence is highly variable and is difficult to be measured with except under laboratory conditions [16, 26].

Toxicity tests have been conducted on copper with a wide range of freshwater plants and the sensitivities are similar to those of animals [25].

5. Conclusions

Perhaps, the purified industrial waste water contains detergents up to the maximum acceptable toxicant concentration (MATC). Although the monitored toxicants concentrations were in limits, the fish mortality was nearly 100% for all variants of the first scheme of testing.

The toxic effect of copper ions were reduced in PIWW perhaps due to the changes in the bioavailability modified under the influence of other impurities, or masked by the influence of detergents. Complexing effects of the test media and a lack of good analytical data make interpretation and application of results difficult.

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