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ECOTOXICOLOGICAL EVALUATION THE EFFECTS OF THE SAFE CONCENTRATION OF WASTEWATER CONTAINING PHENOL ON AQUATIC ECOSYSTEMS

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Abstract. The aim of this study was to identify the toxicity, determine and verify safe concentration of effluents containing phenol to the aquatic ecosystems on the basis of single- and multispecies ecotoxicological bioassays. Synthetic wastewater imitating municipal sewage showed acute toxicity in relation to all bioindicators and belonged to the third toxicity class. The most sensible organism was *Danio rerio,* the most resistance organism was *Desmodesmus quadricauda*. Chronic safe concentration of wastewater containing phenol was 0.63% which corresponded to 0.63 mg/l of phenol. Appointed safe concentration and the one ten times higher than safe were verified in microcosm study, which confirmed that safe concentration did not cause toxic effects. Maximum permissible concentration of phenol in water bodies does not exceed determined concentration in different countries. Proposed research model can be used to determine and verify safe concentrations for aquatic ecosystems of many types of sewage from various industries.

Keywords: phenol, wastewater, toxicity, safe concentration, bioassay, microcosm, water bodies, water pollution.

Introduction

The problem of phenolic water pollution is actual from the second half of the XX century until now in Ukraine and abroad. Phenol is one of the most common pollutants that enter surface water with untreated or insufficiently treated domestic sewage and industrial effluents of oil refining, woodworking, by-product-coking, wood-pulp, paper, plastic, resin and textile industrial enterprises (Michalowicz, Duda 2007).

Concentrations of phenol up to 10 times higher than maximum permissible are observed in numerous Ukrainian water bodies such as Dnieper river and its tributaries Gorin, Desna, Sula, Grouse, Vorskla, Samara, Ingulets which receive industrial wastewater. Nowadays, in conditions of mass urbanization, special risk is arising from the use of contaminated water bodies of urban agglomerations for recreational purposes. This is intensified by additional income of phenol in the issue of summer natural processes. For example, in Lake Vyrlytsia (Kyiv, Ukraine) which partly used for recreational purposes, the content of phenols coming mainly from the industrial zone from time to time exceed maximum permissible concentration in 5 times.

Phenol toxicity relates to two main processes – unspecified toxicity related to hydrophobocity of the individual compound and formation of free radicals. Ability of phenol and its derivatives to alter membrane structure leads to the imbalance of cell environment which results in the cells' death (Hansch *et al.* 2000). Phenol exposure causes the disruption of metabolic system in microorganism, animal and human. It can strongly inhibit the growth of bacteria, algae and mollusks (Gao *et al.* 2006; Huang *et al.* 1996; Park *et al.* 2012). After entering into the fish body, phenol compounds affect the metabolism, survival, growth and reproductive potential of fish (Nahed S. Gad, Amal S. Saad 2008; Hori *et al.* 2006). There are various median lethal concentration LC_{50} of phenol for different fish species. For example, for *Ictalurus punctatus* and *Piaractus mesopotamicus* LC_{50} values are 15.08 and 32.56 mg/l respectively (Moraes *et al.* 2015),

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for *Oreochromis mossambicus* it is 28.49 mg/L (Saha *et al.* 1999), for *Clarius lazera* it is 150 mg/L (Zaki *et al.* 2011). With regard to human health, phenol damages kidneys, liver, muscle, eyes; it irritates skin and causes its necrosis (Bazrafshan *et al.* 2013).

The aim of this study was to determine the safe concentration of effluents containing phenol to the aquatic ecosystems.

1. Previous research on the subject

While there not many studies have been done on toxicity evaluation of wastewater containing phenol, quite a number of ecotoxicological researches were carried out to assess phenol toxicity to different test objects. Among other authors' study of phenol toxicity, there were ones conducted by Załęska-Radziwiłł M., Sheedy B. R., Lazorchak J. M., Grunwald D. J., Pickering Q. H., Pilli A., Hall D., Weeb R., Kaiser K. L., Palabrica V. S. In their researches median lethal concentration LC_{50} and median effective concentration EC_{50} values were obtained for algae *Selenastrum capricornutum* in the range from 224 mg/l to 150 mg/l, *Chlorella vulgaris* – from 370 mg/l to 63 mg/l, rotifer *Brachionus calyciflorus* – from 1200 mg/l to 42 mg/l, crustacean *Daphnia magna* – from 78 mg/l to 10 mg/l, crustacean *Thamnocephalus platyurus* – from 75.5 mg/l to 33 mg/l, crustacean *Artemia salina* – from 260 mg/l to175 mg/l, bacteria *Vibrio fischeri* – from 42 mg/l to 21.1 mg/l (Załęska-Radziwiłł 1997; Sheedy *et al.* 1991; Kaiser, Palabrica 1991; Provisional List 1993).

Toxicity of wastewater from a resin production plant containing phenol to aquatic organisms from different taxonomic groups was studied by Tatjana Tisler and Jana Zagorc-Koncan. Test organisms included mixed bacterial culture, algae *Scenedesmus quadricauda,* crustacean *Dophnia pulex* and fish *Oncorhynchus mykiss*. Toxicity assessment was based on estimation of LC_{50} and EC_{50}. It was found out that EC_{35} of effluents with concentration of phenol 70 mg/l was 100% for mixed bacterial culture, EC_{50} of wastewater with concentration of phenol 40.3 mg/l was 57.5% for *Scenedesmus quadricauda*, EC_{50} of effluents with concentration of phenol 12 mg/l was 17.2% for *Daphnia pulex*, LC_{50} of wastewater with concentration of phenol 12.9 mg/l was 18.5% for *Oncorhynchus mykiss* (Tisler, Zagorc-Koncan 1997). Operation with LC and EC values is suitable for comparison of toxicity but it is not enough for applying to the environment. For decision-making, we should operate with other indicators, such as safe concentration.

2. Scope of the research

The scope of the research included conducting of the following single-species bioassays with representatives of different trophic levels on wastewater containing phenol: algal growth inhibition test with *Desmodesmus quadricauda*, immobilization toxicity test with crustacean *Daphnia magna Straus*, fish *Lebistes reticulatus Peters* and *Danio rerio*, enzymatic tests with *Daphnia magna Straus* and bacteria *Vibrio fischeri*. On the basis of bioassaying results, chronic safe concentrations of studied wastewater were identified. Appointed safe chronic concentration was verified in the aquatic ecosystem model of microcosm type.

3. Materials and methods

3.1. Materials

In the research, there was used synthetic wastewater prepared in laboratory by Weinberger method (Weinberger 1949). Such type of wastewater is representative and universal as it imitates municipal sewage – the composition of domestic wastewater, industrial wastewater and stormwater.

It is comprised of the following ingredients: dry nutrient medium (75 mg/dm^3), peptone (50 mg/dm^3), urea (30 mg/dm^3), sodium acetate (100 mg/dm^3), sodium chloride (30 mg/m^3), potassium chloride (7 mg/dm^3), calcium chloride (7 mg/dm^3), magnesium sulfate (50 mg/dm^3), hydro sodium phosphate (63 mg/dm^3), sodium bicarbonate (168 mg/m^3), soluble starch (100 mg/dm^3), and distilled water. Phenol concentration in the studied wastewater was 100 mg/l.

3.2. Toxicity bioassays

3.2.1. Producers

Growth inhibition test with *Desmodesmus quadricauda* was conducted in accordance with ISO 8692 Standard (2012). Algae in exponential growth phase were added to the mineral medium containing defined concentrations of wastewater. Test consisted of calculation of algae cells number in 1 ml of sample using a microscope before and after 72-hour exposure time and estimation of algae growth-rate reduction by 50% (EC_{50}-72h).

3.2.2. Consumers

One-hour EC_{50} value was calculated as a result of the acute enzymatic test with *Daphnia magna Straus* (Janssen *et al.* 1993). Toxicity evaluation was based on inhibition of galactosidase enzyme activity and as a consequence inhibition of light emission by irreversibly damaged organisms under UV light.

Acute toxicity tests with *Daphnia magna, Lebistes reticulatus Peters* and *Danio rerio* were conducted in accordance with ISO 6341 Standard (2012) and Polish Standard PN-90/C-04610.04 (1990). Organisms were exposed to different concentrations of wastewater for a certain time. Test was based on estimation of organisms' immobilization and survival after 48 h for crustacean and 96h for fishes. Data are represented as LC_{50}.

3.2.3. Decomposers

The LUMIStox acute toxicity test with bacteria *Vibrio fischeri* was carried out using LUMIStox measuring instrument model 1.07 in accordance with methodology

included in the implementing instruction (LUMIStox 1994). Test was based on inhibition of the luciferase enzyme and as a consequence reduction of bacteria light emission intensity. The assessment of the bioluminescence inhibition was conducted after 30 min of bacteria exposure to different concentrations of wastewater.

3.3. Calculation methods

The percentage of algae growth rate inhibition $I\mu_i$ was calculated by the formula

$$I_i = \frac{\mu_c - \mu_i}{\mu_c} \times 100\% , \quad (1)$$

where μ_c – average growth rate of algae in the control sample, μ_i – average growth rate of algae in the given concentration that was determined from the obtained number of algae cells in 1 cm^3 at time t_0 (N_0) and the number of algae cells in 1 cm^3 at time t (N_n), using equation:

$$\mu_i = \frac{\ln N_n - \ln N_0}{t_n} . \quad (2)$$

For all conducted bioassays, except LUMIStox test, lethal and effective concentrations $LC(EC)_{50}$ were determined by probit analysis with 95% confidence intervals (Weber 1972). LUMISsoft II software was used for obtaining EC_{50} during 30 min of exposure time for test with bacteria *Vibrio fischeri.*

For *NOEC* (No Observed Effect Concentration) calculation was done extrapolation from the results of acute toxicity using Acute to Chronic Ratio *ACR* = 10 according to the formula

$$NOEC = \frac{LC(EC)_{50}}{ACR} . \quad (3)$$

Acute toxicity units TU_a were obtained from the equation:

$$TU_a = \frac{100}{LC(EC)_{50}} . \quad (4)$$

Chronic toxicity units TU_c were determined in accordance with formula

$$TU_c = \frac{100}{NOEC} . \quad (5)$$

Estimation of safe concentration (*SC*) with regard to the chronic toxicity is based on the assumption that it does not exceed *CCC* – Criteria Continuous Concentration – the highest concentration that does not cause toxic effects in zone of mixing with the water of reservoir in the period of 4 days (EPA/5O5/2-90-001:1991). The safe concentration of studied wastewater is calculated by the equation

$$SC = \frac{CCC}{TU_{c,max} \cdot RPMF} \times 100\% , \quad (6)$$

where $TU_{c,\,max}$ is the highest value of the units of toxicity TU_c, *RMPF* is the reasonable potential multiplying factor which depends on the coefficient of data variation, confidence level and probability. Coefficient of variation was calculated on the basis of standard deviation σ and average value of chronic toxicity units $TU_{c,m}$:

$$CV = \frac{\sigma}{TU_{c,m}} . \quad (7)$$

RPMF was read for variation coefficient *CV* = 0.4, confidence level 95% and probability of 95%. *CCC* is accepted as $1TU_c$.

All values of $LC(EC)_{50}$ as well as *SC* value are expressed as volume percentage concentrations.

3.4. Microcosm study

Verification of calculated chronic safe concentration in microcosm study was conducted in 12 five-liter aquariums:

- 4 control aquariums,
- 4 aquariums containing safe concentration of the researched wastewater,
- 4 aquariums containing ten times higher than safe concentration of wastewater.

Each aquarium was filled with water treated in biofilter and settled by the following organisms: algae culture *Desmodesmus quadricauda* and *Selenastrum capricornutum*, duckweed *Lemna minor*, crustaceans *Daphnia magna Straus*. Model ecosystems with introduced organisms were subjected to weekly adaptation with aeration and light, and then appropriate concentrations of wastewater were added. Hydrobiological, microbiological and chemical parameters were assessed for analysis of ecosystems development dynamics during 4 weeks with 7-day intervals.

3.4.1. Hydrobiological parameters

Hydrobiological parameters included microscopic estimation of *Daphnia magna Straus* number using *Kolkwitz's chamber,* microscopic analysis of algae (*Desmodesmus quadricauda* and *Selenastrum capricornutum*) development, and assessment of leaves number of duckweed *Lemna minor*. Data are represented as cells/ml for algae and as units for crustaceans and duckweed development.

3.4.2. Microbiological parameters

Microbiological parameters included determination of total number of bacteria by Koch method by submerged seeding on nutrient agar medium (Grabińska-Łoniewska 1999). Results are represented as cfu/ml units.

3.4.3. Chemical parameters

Chemical parameters included pH determination by electrometric method, ammonia nitrogen determination by direct *nesslerization method,* nitrite and nitrate nitrogen determination by colorimetric method, dissolved orthophosphate determination by colorimetric method, chloride determination by titration method, chemical oxygen demand (COD) determination by *dichromate reflux method* (Clescerl *et al.* 1999).

4. Results and discussion

The results of single-species toxicity tests on wastewater containing phenol are presented in Table 1.

Results of bioassays indicate harmful effects of wastewater containing phenol on all test organisms. $LC(EC)_{50}$ values vary with regard to different test objects due to different sensitivity of organisms. The most sensible organism is *Danio rerio,* LC_{50} for which is 10.7%. The most resistance organism is algae *Desmodesmus quadricauda* with LC_{50} 33.6%. Toxicity assessment with regard to the acute toxicity on the basis of TU_a values according to Persoon *et al.* (2003) was made on basis of data obtained from single-species bioassays. Researched wastewater is referred to III class of toxicity and shows acute toxicity.

Using $LC(EC)_{50}$ values No Observed Effect Concentrations and chronic toxicity units TU_c were obtained. TU_c data were used for calculation of safe concentration of studied wastewater with regard to the chronic toxicity. Found safe concentration is 0.63% which corresponds to 0.63 mg of phenol per liter.

The next stage of research consisted in microcosm study, where there were verified the safe concentration and the one 10 times higher than safe. Microcosm study shows effects of wastewater on organisms of different organizational levels as well as interactions among the different components resulting in indirect exposure effects of both functional and structural nature (Oskarsson *et al.* 2012).

Figures 1–4 present dynamics of microbiological and hydrobiological parameters during examination time in microcosm study.

Table 1. Results of single-species bioassays and toxicity assessment of wastewater

No.	Test organism	Type of test	Duration, [h]	LC(EC)50, [%] (95% confidence interval)	TU_a	Toxicity class and assessment	*NOEC*	TU_c
1	*Desmodesmus quadricauda*	growth inhibition test	72	33.6 (31.9–35.1)	2.98	III, acute toxicity	3.36	29.8
2	*Vibrio fischeri*	enzymatic tests	0.5	28.5 (27.5–30.0)	3.51	III, acute toxicity	2.85	35.1
3	*Lebistes reticulatus*	Immobilization test	96	21.3 (19.9–22.6)	4.69	III, acute toxicity	2.13	47
4	*Daphnia magna Straus*	survival test	48	19.6 (18.4–20.8)	5.10	III, acute toxicity	1.96	51
5	*Daphnia magna Straus*	enzymatic test	1	19.6 (18.2–20.6)	5.10	III, acute toxicity	1.96	51
6	*Danio rerio*	immobilization test	96	10.7 (9.9–11.5)	9.35	III, acute toxicity	1.07	93.5

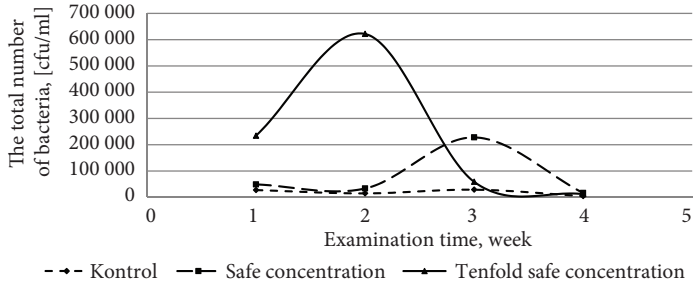

Figure 1. Dynamics of changes in the total number of bacteria in microcosm study

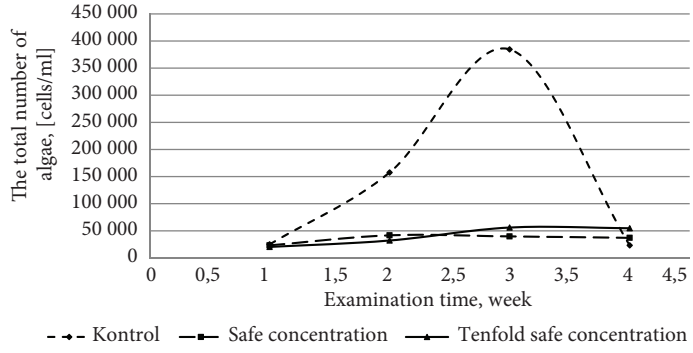

Figure 2. Dynamics of total number of algae development in microcosm study

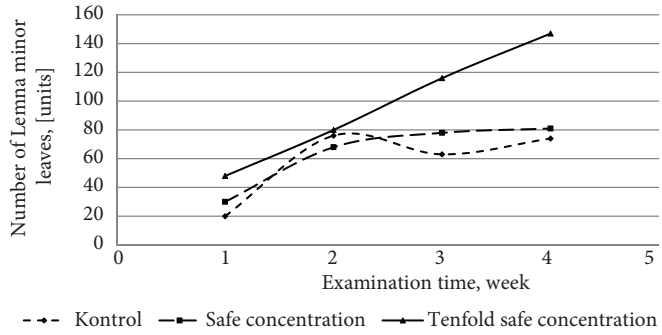

Figure 3. Dynamics of changing the leaves number of *Lemna minor* in microcosm study

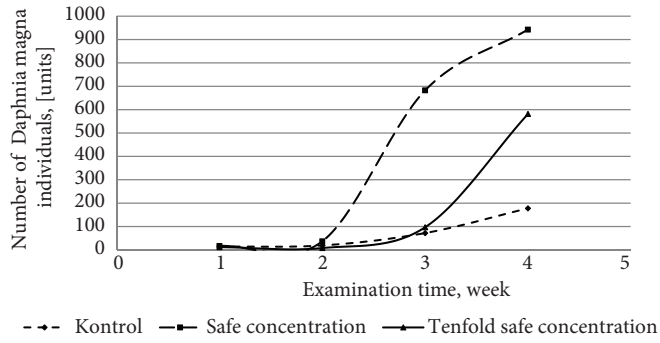

Figure 4. Dynamics of changing the number of *Daphnia magna Straus* in microcosm study

Changing of chemical parameters during examination time in microcosm study is presented in Table 2.

Table 2. Chemical parameters in microcosm study

Indicator	Examination time	Concentration		
		Control	Safe concentration	Tenfold safe concentration
1	2	3	4	5
pH	Week 1	8.8	9.0	8.6
	Week 2	8.5	8.7	9.8
	Week 3	8.5	8.3	9.26
	Week 4	8.5	8.5	8.87
COD, $\left[\frac{mg\ O_2}{dm^3}\right]$	Week 1	105.6	110.4	244.0
	Week 2	86.4	124.8	163.2
	Week 3	126.9	107.4	112.2
	Week 4	104.7	99.2	119.0
Ammonia nitrogen NH_4^+, $\left[\frac{mgN–NH_4^+}{dm^3}\right]$	Week 1	0.14	0.16	0.28
	Week 2	0.04	0.06	0.15
	Week 3	0.05	0.08	0.14
	Week 4	0.10	0.11	0.14
Nitrite nitrogen NO_2^- $\left[\frac{mgN–NO_2^-}{dm^3}\right]$	Week 1	0.023	0.050	0.050
	Week 2	0.005	0.007	0.007
	Week 3	0.004	0.006	0.006
	Week 4	0.011	0.050	0.006

End of Table 2

1	2	3	4	5
Nitratenitrogen NO_3^-, $\left[\frac{mgN-NO_3^-}{dm^3}\right]$	Week 1	1.3	1.1	1.0
	Week 2	1.2	1.1	0.9
	Week 3	0.9	1.0	1.2
	Week 4	1.1	1.2	1.0
Dissolved orthophosphate PO_4^{3-}, $\left[\frac{mgPO_4^{3-}}{dm^3}\right]$	Week 1	2.93	3.75	4.7
	Week 2	4.03	3.51	3.32
	Week 3	5.97	6.15	6.03
	Week 4	4.77	5.1	4.53
Chloride Cl^-, $\left[\frac{mg\ Cl^-}{dm^3}\right]$	Week 1	211	210	228
	Week 2	248	246	269
	Week 3	275	270	285
	Week 4	280	286	308

At the beginning of the experiment the population of bacteria in the sample with tenfold higher than safe concentration of wastewater is much greater than in other trials. This difference has developed in the period of a week adaptation of organisms to the environment independently of the added wastewater. Dynamics of bacteria population development in this sample is proportional to the availability of nutrients: at the second week bacterial growth is observed while concentrations of NH^{4+}, $PO4^{3-}$,

COD are decreased. It means that microorganisms consume carbon compounds, phosphorus and ammonium nitrogen to build biomass. At the 3rd week decrease in the number of bacteria occurs after depletion of nutrients.

In microcosm with safe concentration of researched wastewater the amount of nutrients was less due to the smaller amount of introduced sewage, which was resulted in the less intense growth of microorganisms. The reason for bacteria number development in this sample between 2nd and 3rd week was the growth of crustacean *Daphnia magna* and increased amount of their wastes, which were the source of organic compounds for bacteria.

Growth of algae is observed in each sample, but most intensively it had been developed in the sample with tenfold higher than safe concentration of sewage. In the presence of such large amount of nutrients, there was an intensive growth of this algae population, reaching a maximum in the third week, and later their number began to decrease due to lack of nutrients. The same situation we observe in the sample with safe concentration of wastewater between 1st and 2nd week.

After observing the changes in algae and bacteria populations the toxic effects caused by wastewater containing phenol is not recognized. On the contrary, in the sample with sewage concentration tenfold higher than safe for most organisms we observe a significant stimulating effect of the added pollutant. It is most evident in the case of autotrophs.

Daphnia magna population increases with the enhancement of food availability (algae growth as well as nitrogen and phosphorus compounds).

Duckweed used for growth and reproduction contained in the environ-aqueous medium nitrogen and phosphorus.

Chloride ion concentration changes in a stable manner and it is not correlated with changes in populations and other chemicals. Increasing of chloride ions concentration indicates ongoing mineralization.

Conclusions

On the basis of single-species ecotoxicity tests results, it is determined that the synthetic sewage by Weinberger method with the addition of phenol showed acute toxicity in relation to all bio-indicators and belonged to the third toxicity class according to the criteria developed by Persoon *et al.* (2003).

Based on single species toxicity tests, chronic safe concentration of wastewater containing phenol 0.63%, which corresponds to 0.63 mg/l of phenol, does not cause toxic effects in the test environment – microcosm.

Tenfold higher than safe concentration of wastewater caused a short-term effect of stimulation of organisms growth. After 4 weeks of research, the microbiological, most of chemical and some hydrobiological parameters have the tendency to approach to similar parameters measured in control. This indicates a self-purification of test ecosystems.

Safe concentration of phenol to aquatic ecosystems, calculated in accordance with Załęska-Radziwiłł extrapolation model, is 0.59 mg/l, which differs slightly from data we have obtained for wastewater containing phenol (Załęska-Radziwiłł 1997). This indicates that the toxicity of the studied sewage to the aquatic ecosystems mainly caused by the presence of phenol and insignificantly depends on other components.

According to the regulatory documents in Ukraine, the maximum allowable concentration of phenol in surface water is 0.001 mg/l (Sanitary rules and regulations 1988). In EU countries, the maximum concentration of phenol allowed in drinking water is 0.0005 mg/l (Council Directive 98/83/EC). Concentration of phenol for all classes of surface water in Poland should not exceed 0.01 mg/l (Dz. U. 1482:2014). In all cases, the maximum allowable concentration of phenol in water bodies does not exceed determined safe concentration on the basis of single-species toxicity tests and verified in the microcosm study.

Scientific articles mainly focused on treatment of phenolic sewage, but there is a lack of such comprehensive ecotoxicological studies of wastewater, including single- and multi-species tests (Wiessner *et al.* 2014; Pishgar *et al.* 2014; Riauka *et al.* 2006; Mohammadi *et al.* 2015). The proposed research model can be used to determine and verify safe concentrations not only for wastewater with phenol, but also originating from other industrial sources, which may contribute greatly to the protection of aquatic ecosystems.

Disclosure statement

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