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# Mode of joint action response to binary mixtures of three refined petroleum products by Nile tilapia *Oreochromis niloticus* fingerlings

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The toxicities of three refined petroleum products and their binary mixtures based on predetermined and equitoxic ratios of 1:1, 1:6 for petrol-kerosene; 1:1, 1:2 for petrol-diesel and 1:1, 1:3 for diesel-kerosene respectively were evaluated against the fingerlings of *Oreochromis niloticus*, in laboratory bioassays. The interactions between binary mixtures showed significant departures from the action of the individual constituent compound when acting singly. On the basis of synergistic ratios (SRs) and concentration-addition models (RTU) used for the joint action evaluations, the interactions between the constituent toxicants in the various test proportions of the mixtures were largely in conformity with the model of synergism ( $SR > 1$  and  $RTU > 1$ ) except for petrol-kerosene (1:1) which was additive ( $SR = 1$ ). In most of the test combinations petrol was found to consistently increase the toxic effect of kerosene and diesel. The joint action toxicity studies provides a means of identifying the integrated impact of chemical mixtures on different levels of biological function and could make a valuable and viable addition to routine management protocols for protecting fragile aquatic ecosystems.

**Key words:** Joint action, binary mixtures, refined petroleum products, synergism.

## INTRODUCTION

Crude oils and their refined petroleum products are essentially a complex mixture of hydrocarbons, which may contain cancer-causing polycyclic aromatic fractions and other toxic substances (Afolabi et al., 1985). While the components of these petroleum products share some similar physical and chemical characteristics, the toxicological properties of these components can be quite different (Anderson et al., 1974; Ariens et al., 1976). For this reason, where generic standards are not available or when toxic components are known to be present, it is usually necessary to determine the atmospheric concentration of each of the major or toxic components and compare these with the appropriate individual exposure standard.

As a result of the potential toxic nature of refined petroleum products it is necessary to establish a dose-response relationship when sensitive species are exposed to varying concentrations in order to extrapolate lethal concentrations ( $LC_{50}/LD_{50}$  values) and sublethal

concentrations. These derived toxicity indices are used as a tool in petroleum products pollution identification, control and management particularly in the establishment of environmental safety limits and toxicity scales or ranking orders of the pollutants (Mason, 1992). Such scales, being relevant in making possible the choice of environmental compounds with the lowest risk of biological damage. Ottaway (1976) and Chukwu (2003, 2004), showed that on the basis of 96-h  $LC_{50}$  values, petrol was more toxic to crustaceans and teleost fish, followed by kerosene and diesel oils respectively. However, such single action studies are not likely to describe accurately, the effects of pollutants which act simultaneously against exposed organisms in natural habitats.

Some of the major challenges in ecotoxicological evaluation of risk in environmental management includes the analysis of the effects of the physico-chemical environment on the speciation and uptake of pollutant chemicals and inherent inter-individual and inter species differences in vulnerability to toxicity and the toxicity of complex mixtures (Moore et al., 2004). Contaminants are seldom present as a single chemical and usually comprise a complex mixture (Handy et al., 2002). The toxicity of

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xenobiotics from such mixtures is poorly understood and questions of whether components of the mixtures influence the uptake and biotransformation of other components have not been seriously addressed (Kortenkamp, 1998).

Recent information on joint action toxicity of mixtures of components has shown that the types of interactions exhibited by components of mixtures are largely dependent on the proportion of their occurrence in the mixture (Otitolaju, 2005).

In view of the above mentioned, the objectives of this study were to evaluate the interacting effects of combinations of mixtures of refined petroleum product against the Nile tilapia, *Oreochromis niloticus*, fingerlings based on predetermined equitoxic ratio that depict their individual 96hLC<sub>50</sub> values.

## MATERIALS AND METHODS

### Source and maintenance of test organisms

Live fingerlings of *O. niloticus* (mean weight: 1.93 g, mean length: 3.57 cm) used for this study were collected from Animashaun farms in Badagry, Lagos state. The fishes were transported to the laboratory in oxygenated polythene bags.

### Acclimatization of test organism

In the laboratory, the fishes were kept in glass holding tanks (50 x 30 x 30cm) which contained dechlorinated tap water and acclimatized for a period 7 days. The water was continuously aerated with 220 V air pumps and changed once in 2 days. The fishes were fed twice daily during the period of acclimatization with commercial fish feed pellets at 5% body weight. The fishes were fed every day until a day preceding the bioassay test. Stocking and experimentation was carried out under ambient laboratory conditions (temperature 27± 3°C, relative humidity 79±2%) in accordance with guidelines for bioassay techniques (APHA, 1985).

### Test solutions

The petrol, kerosene and diesel used for this experiment were bought from AP Filling station in the University of Lagos, Akoka, Yaba, Lagos, Nigeria. A predetermined volume of each toxicant was measured out and made up to a given volume to obtain solutions of required concentration. The dilution was carried out in glass tanks. The solutions produced were mixed thoroughly by stirring with glass rod.

### Bioassay containers

The bioassays were carried out in glass tanks (22 x 15 x 18 cm). These glass tanks were preferred to plastic containers as they minimize absorption of toxicants and prevent risk of corrosion and chemical reactions. Some plastics are known to react with some crude oil components (Don – Pedro, 1989).

### Application of toxicant to test media

Dechlorinated tap water was measured using measuring cylinder

into clean, dry bioassay containers, and a predetermined volume of petrol, kerosene and diesel was added into the water to make it up to 2000 ml (total volume of test media) to achieve the desired test concentration.

### Assessment of quantal response

The fish, *O. niloticus* were assumed to be dead if there was no movement of appendages, opercular and mouth or failed to respond to the touch of forceps /glass rod.

### Single action toxicity tests of refined petroleum products against *Oreochromis niloticus* fingerlings

Ten fishes of similar sizes in three replicates were introduced randomly into the test media in bioassay containers. A total of 30 fish fingerlings were exposed per treatment including untreated control (dechlorinated tap water). The test animals were exposed to varying concentrations of refined petroleum products as follows:

Petrol - 0.55, 0.56, 0.57, 0.58, 0.59 and 0.60 ml/L,  
Kerosene - 3.00, 3.20, 3.40, 3.60, 3.80 and 4.00 ml/L  
Diesel -0.50, 0.70ml, 0.90, 1.10, 1.30 and 1.50 ml/L

Mortality was assessed once every 24 h for 4 days (96 h)

### Joint-action toxicity of binary mixtures petrol, diesel and kerosene against *Oreochromis niloticus* fingerlings

A series of bioassays similar to those described for single action tests were carried out but in this instance, the acclimatized animals of similar sizes were exposed to the different concentrations of predetermined ratios (1:1) and equitoxic ratios (1:6, 1:2 petrol-kerosene/diesel; 1:3 diesel-kerosene). At each predetermined concentration of a mixture to be tested, the proportion of each constituent toxicant dictated by the predetermined ratio of the mixture was computed and measured out into a conical flask and was made up to the required test media volume by adding dechlorinated tap water as diluent. The test media was then mixed thoroughly by stirring with glass rod for 3 mins before the mixture was transferred at the desired concentration into the appropriate bioassay container (Otitolaju, 2005). The fish fingerlings were exposed to varying concentrations of the mixtures as follows:

Petrol and Kerosene (ratio: 1:1) - 0.50, 0.52, 0.54, 0.56, 0.58 and 0.60 ml/L.  
Petrol and Kerosene (ratio: 1:6) - 0.28, 0.32, 0.36, 0.40, 0.44 and 0.48 ml/L.  
Petrol and Diesel (ratio: 1:1) - 0.50, 0.52, 0.54, 0.56, 0.58 and 0.60 ml/L.  
Petrol and Diesel (ratio: 1: 2) - 0.30, 0.32, 0.34, 0.46, 0.48 and 0.40 ml/L..  
Diesel and Kerosene (ratio: 1:1) - 0.50, 0.70, 0.90, 1.10, 1.30 and 1.50 ml/L.  
Diesel and Kerosene (ratio: 1:3) - 0.80, 0.84, 0.88, 0.92, 0.96 and 1.00 ml/L.

This set up was replicated to eliminate possible errors due to handling, differences in size and weight and other intrinsic physiological imbalances in the test fishes. Mortality was assessed once every 24 h for 4 days (96 h). Care was taken to minimize the stress on the fish by using a hand net to collect and drop them carefully into the bowls.

### Statistical analysis

Toxicological dose-response data involving quantal response (mortality) were analysed by probit analysis (Finney, 1971) based on a computer program written by Ge Le Pattourel, Imperial College, London, as adopted by Don-Pedro (1989). The indices of toxicity derived from this analysis were:

LC<sub>50</sub> = Median lethal concentration that causes 50% response (mortality) of exposed organisms.

LC<sub>95</sub> = Lethal concentration that causes 95% response (mortality) of exposed organisms.

LC<sub>5</sub> = Sublethal concentration that causes 5% response (mortality) of exposed organisms and their 95% confidence limits (CL).

TF = Toxicity factor for relative potency measurements, e.g., 96-h LC<sub>50</sub> of a compound / 96-h LC<sub>50</sub> of another compound tested against same species.

One Way Analysis of Variance (ANOVA) and comparison of means by Student Newman-Keuls (SNK) test were used to test for statistical differences in the results of 96 h toxicity tests.

### Analysis of data and measurement of joint-action toxicity of binary mixture of test compounds

The two models used for the analysis of the joint-action toxicity tests were Synergistic ratios (S.R.) Model after Hewlett and Packard (1969) and the concentration-addition model of Anderson and Weber (1975) with slight modification (relative toxic units; Otitoloju, 2005).

**Model A:** Measurement of Joint-action toxicity by synergistic ratios (SRs). SR is defined after Hewlett and Plackett (1969) is:

LC<sub>50</sub> of a Chemical Acting Alone  
LC<sub>50</sub> of Chemical + Additive (Mixture)

Where; SR = 1 describes the additive action  
SR < 1 describes antagonism  
SR > 1 describes synergism

**Model B:** Concentration – addition model (Anderson and Weber, 1975). This model assumes that when similarly acting toxicants are mixed in any proportion they will add together to give the observed response in evaluating the joint-action, a predicted response in evaluating the joint-action, a predicted response value (s) is derived by summing up the LC<sub>50</sub> values of the separate toxicants according to the proportion of their contribution in the mixture. The predicted LC<sub>50</sub> value (s) is then compared to the observed LC<sub>50</sub> value of the mixture to classify the type of interaction among the components of the mixture as follows:

- i) Additive if the observed LC<sub>50</sub> value of the mixture is equal to the predicted LC<sub>50</sub> value.
- ii) Synergistic if the observed LC<sub>50</sub> value of the mixture is less than the predicted LC<sub>50</sub> value.
- iii) Antagonistic if the observed LC<sub>50</sub> value of the mixture is greater than the predicted LC<sub>50</sub> value.

The relationship of derived LC<sub>50</sub> values to predicted LC<sub>50</sub> (RTU) is estimated as:

RTU = Predicted LC<sub>50</sub> value / Experimentally derived LC<sub>50</sub>  
Where RTU = relative toxic unit. RTU = 1 describes additive action; RTU < 1 describes antagonism; and RTU > 1 describes synergism.

## RESULTS

### Single-action toxicity of refined petroleum products against *Oreochromis niloticus* fingerlings

The analysis of dose response data of refined petroleum products when tested against the tilapia fingerlings revealed that the derived toxicity indices (96-h LC<sub>50</sub> values) recorded for petrol; kerosene diesel were , 0.571, 3.420 and 1.042 ml/L, respectively (Table 1). On the basis of 96-h LC<sub>50</sub> petrol was the most toxic petroleum fraction followed by diesel and kerosene in a descending order of toxicity. Petrol was significantly (no overlap in 95% confidence limits of 96-h LC<sub>50</sub> values) more toxic than diesel and kerosene. Diesel was also significantly (no overlap in 95% confidence limits of 96-h LC<sub>50</sub> values) more toxic than kerosene. Computed toxicity factors (ratios of 96-h LC<sub>50</sub> values) revealed that petrol was about 1.82 times and 5.99 times more toxic than diesel and kerosene respectively when tested against *O. niloticus* while diesel is 3.28 times more toxic than kerosene (Table 1).

### Joint action toxicity of binary mixtures of refined petroleum products against *Oreochromis niloticus* fingerlings

The analysis of the concentration-mortality data for the binary mixtures of refined petroleum products revealed that the 96-h LC<sub>50</sub> values recorded for petrol and kerosene at ratio 1:1; 1:6 were 0.548 and 0.408 ml/L; petrol and diesel at ratio 1:1; 1:2 were 0.532 and 0.350 ml/L; diesel and kerosene at 1:1; 1:3 were 0.946 and 0.853 ml/L, respectively (Table 2). Therefore, the binary mixtures of petrol and diesel based on equitoxic ratio of 1:2 (96-h LC<sub>50</sub> 0.350 ml/L) was the most toxic while the diesel kerosene mixture based on predetermined ratio of 1:1 was the least toxic (96-h LC<sub>50</sub> 0.946 ml/L).

### Petrol and kerosene mixtures

On the basis of 96-h LC<sub>50</sub> values , the binary mixtures of petrol and kerosene based on ratios (1:1, 1:6) was found to be significantly (no overlap in 95% confidence limits of 96-h LC<sub>50</sub> values) more toxic against *O. niloticus* than petrol and kerosene respectively when acting singly against the test animal (Table 2). When the same set of data dependent on the 96-h LC<sub>50</sub> values, were subjected to additional analysis by being substituted into MODEL A (SR), the derived SR (SR<sup>1</sup>) with reference to petrol at ratio 1:1 was approximately 1 (SR = 1) tending to agree with the model of additive action (Table 2) whereas the SR for ratio 1:6 with reference to petrol was above 1 (SR>1) indicating synergism. The derived SR (SR<sup>2</sup>) with reference to kerosene at ratios (1:1: 1:6) were above 1

**Table 1.** Relative toxicity of petrol, kerosene and diesel against *Oreochromis niloticus* fingerlings.

Time (h)	Treatment	LC <sub>5</sub> (95% C.L)	LC <sub>50</sub> (95% C.L)	LC <sub>96</sub> (95% C.L)	Slope + S.E	D.F	Probit line equation	TF <sub>1</sub>	TF <sub>2</sub>
24	Petrol	0.573 (0.553-0.581)	0.618 (0.605-0.663)	0.667 (0.636-0.787)	49.795+14.204	4	Y=10.407+49.795x	1	
	Kerosene	3.647 (3.383-3.753)	4.142 (4.001-4.567)	4.704 (4.367-6.037)	29.770+8.371	4	Y=-18.374+29.770x	6.70	
	Diesel	1.516	2.600	4.460	7.017+3.686	4	Y=-2.912+7.017x	4.21	
48	Petrol	0.558 (0.541-0.566)	0.601(0.593-0.615)	0.647 (0.627-0.694)	51.314+10.432	4	Y=11.362+51.314x	1	
	Kerosene	3.384 (3.219-3.483)	3.842 (3.766-3.945)	4.360 (4.183-4.707)	29.891+4.822	4	Y=-17.471+29.891x	6.55	
	Diesel	1.000 (0.631-1.169)	2.356 (1.866-5.297)	5.553 (3.259-40.65)	4.417+1.313	4	Y=-1.644+4.417x	3.92	
72	Petrol	0.547 (0.532-0.556)	0.587(0.582-0.594)	0.629 (0.616-0.656)	54.400+9.054	4	Y=12.594+54.400x	1	
	Kerosene	3.196 (3.044-3.296)	3.661 (3.591-3.738)	4.193 (4.049-4.437)	27.893+3.707	4	Y=-15.720+27.893x	6.24	
	Diesel	0.734 (0.542-0.859)	1.451(1.329-1.644)	2.870 (2.297-4.464)	5.553+0.972	4	Y=-0.898+5.553x	5.83	
96	Petrol	0.544 (0.534-0.551)	0.571(0.567-0.575)	0.600 (0.593-0.610)	78.343+10.038	4	Y=19.048+78.343x	1	
	Kerosene	2.956 (2.799-3.062)	3.420 (3.348-3.489)	3.957 (3.831-4.157)	25.979+3.218	4	Y=-13.873+25.979x	5.99	3.28
	Diesel	0.615 (0.492-0.706)	1.042 (0.962-1.116)	1.764 (1.577-2.106)	7.187+0.946	4	Y=-0.127+7.187x	1.82	1

LC = Lethal concentration; C.L = confidence limits; and TF = toxicity factor.

TF<sub>1</sub> = 24-h LC<sub>50</sub> / 24 or 48 or 72 or 96 h value.

TF<sub>2</sub> = 96-h LC<sub>50</sub> of kerosene / 96-h LC<sub>50</sub> of diesel.

(SR >1) also indicating synergism.

Further analysis of the joint- action toxicity data based on the experimentally observed and depicted 96-h LC<sub>50</sub> values by the Concentration-Additional model (Model B) also revealed that interactions between petrol and kerosene mixtures in ratios (1:1, 1:6) is in agreement with the model of synergism (RTU>1) although with varying levels of fit (Table 2.)

**Petrol and diesel mixtures**

On the basis of 96-h LC<sub>50</sub> values, the petrol and diesel mixtures based on ratios (1:1, 1:2) was found to be significantly (no overlap in 95% confidence limits of 96-h LC<sub>50</sub> values) more toxic against *O. niloticus* than petrol and diesel respectively when acting singly against the test animal on the 96-h LC<sub>50</sub> values were subjected to addi-

(SR), the derived SR (SR<sup>1</sup>) with reference to petrol at ratios 1:1 and 1:2 were above 1 (SR > 1) tending to agree with the model of synergism. The derived SR (SR<sup>2</sup>) with reference to diesel at ratios (1:1: 1:2) were above 1 (SR >1) also indicting synergism.

Further analysis of the joint- action toxicity data based on the experimentally observed and depicted 96-h LC<sub>50</sub> values by the Concentration-Additional model (Model B) also revealed that interactions between petrol and diesel (1:1, 1:2) were in agreement with the model of synergism (RTU>1) as observed with MODEL B although with varying levels of fit (Table 2).

**Diesel and kerosene mixtures**

On the basis of 96-h LC<sub>50</sub> values, the diesel and kerosene mixtures based on test of the two ratios

(1:1, 1:3) was found to be significantly (no overlap in 95% confidence limits of 96-h LC<sub>50</sub> values) more toxic against *O. niloticus* than diesel and kerosene respectively when acting singly against the test animal (Table 2 )When the same set of data dependent on the 96-h LC<sub>50</sub> values were subjected to additional analysis by being substituted into Model A (SR), the derived SR (SR<sup>1</sup>) with reference to diesel at ratios 1:1 and 1:3 were above 1 (SR > 1) tending to agree with the model of synergism. The derived SR (SR<sup>2</sup>) with reference to kerosene at ratios (1:1: 1:3) were also above 1 (SR >1) indicting synergism.

Further analysis of the joint- action toxicity data based on the experimentally observed and depicted 96-h LC<sub>50</sub> values by the Concentration-

Additional model (Model B) also revealed that interactions between petrol and diesel mixtures in ratios (1:1, 1:3) were in agreement with the model of synergism (RTU>1) as observed with Model A

**Table 2.** Analysis (based on concentration addition model and synergistic ratios models) of the 96 h LC<sub>50</sub> Value of refined petroleum products when acting singly or jointly against *Oreochromis niloticus* fingerlings.

Treatment	Predicted 96-h LC <sub>50</sub> (95% CL) (ml/L)	Experimentally observed 96-h LC <sub>50</sub> (95% CL) (ml/L)	RTU	SR <sup>1</sup>	SR <sup>2</sup>
<b>Petrol/kerosene</b>					
Mixture portraying environmental occurrence 1:1	1.996(1.957-2.033)	0.548(0.540-0.556)	3.64	1.04	6.24
Equitoxic mixture 1:6	0.571(0.559-0.580)	0.408(0.391-0.427)	1.40	1.40	8.38
Petrol alone	-	0.571(0.567-0.575)			
Kerosene alone	-	3.420(3.348-3.489)			
<b>Petrol/diesel</b>					
Mixture portraying environmental occurrence 1:1	0.807(0.765-0.846)	0.532(0.521-0.541)	1.52	1.07	1.96
Equitoxic mixture 1:2	0.537(0.510-0.564)	0.350(0.341-0.358)	1.53	1.63	2.98
Petrol alone	-	0.571(0.567-0.575)			
Diesel alone	-	1.042(0.962-1.116)			
<b>Diesel/kerosene</b>					
Mixture portraying environmental occurrence 1:1	2.231(2.155-2.303)	0.946(0.861-1.038)	2.36	1.10	3.62
Equitoxic mixture 1:3	1.091(1.078-1.151)	0.853(0.820-0.876)	1.28	1.22	4.01
Diesel alone	-	1.042(0.962-1.116)			
Kerosene alone	-	3.420(3.348-3.489)			

CL. 95% confidence limit; RTU, relationship of experimentally observed LC<sub>50</sub> to predicted LC<sub>50</sub>: (predicted 96-h LC<sub>50</sub> value) / (observed 96-h LC<sub>50</sub> value). RTU = 1 indicates additive action; > 1 indicates synergism; < 1 indicates antagonism.

SR<sup>1</sup>, SR<sup>2</sup> = synergistic ratio of the 1<sup>st</sup> and 2<sup>nd</sup> toxicant. SR = (LC<sub>50</sub> value of the toxicant acting alone) / (LC<sub>50</sub> value of the mixture).

SR<sup>1</sup>/ SR<sup>2</sup> = 1 indicates additive action; > 1 indicates synergism; < 1 indicates antagonism.

although with varying levels of fit (Table 2).

## DISCUSSION

The results of the joint action toxicity tests of the binary mixtures of petrol, kerosene and diesel based on predetermined ratio(1:1) portraying possible environmental occurrence and equitoxic ratio of single action toxicity against *O. niloticus* were found to be significantly more toxic than when the compounds were acting alone. The joint- action toxicity evaluations of binary mixtures of refined

petroleum product (petrol-kerosene, petrol-diesel and diesel-kerosene) in different ratios against *O. niloticus* revealed that interactions between the constituent petroleum compounds in the mixtures were largely synergistic except the mixture of petrol and kerosene in predetermined ratio 1:1 which was observed to be additive in action with reference to petrol. Similar toxicological synergistic interactions in joint-action of different test ratios of toxicant mixtures compared to single action toxic effect have been documented in the literature (Khangarot et al., 1982; Parrot and Sprague, 1993; Otitolaju, 2003). Otitolaju (2003)

reported that the joint-action interaction between heavy metals which are prominent in effluent of some industrial establishments in Lagos State, Nigeria and Lagos lagoon sediments against benthic animals, *Tympanotonus fuscatus*, *Clibanarius africanus* and *Sesarma huzardi* of the Lagos lagoon were in conformity with the model of syner-synergism when the test mixtures were prepared on the basis of equitoxic ratios.

The realisation that synergistic interactions could result when pollutants occur in mixtures underscores the need for re-evaluation of the ecological relevance of safety limits in use in some

developing countries where most of the presented safe limits are usually an importation/ adoption of established safe limits of other developed countries or agencies. The results obtained in this study therefore imply that in order to set or review water quality criteria in developing countries; it would be necessary to adopt concentrations of pollutant mixtures, which are more relevant to the nations water bodies and the aquatic biota.

There was a gradient of synergism from slight synergism to strong synergism. The interaction between the mixtures of petrol and kerosene (ratio 1:6), petrol and diesel (ratio 1:1, 1:2) with reference to petrol is slightly more than 1 and less than 2 indicating slight synergism whereas the SR values recorded with reference to kerosene (ratio 1:6) and diesel (ratio 1:2) are above 2 indicating strong synergism. This clearly shows that petrol is most toxic and contributes most of the toxicity in the mixture. Therefore the toxicity of kerosene and diesel is greatly enhanced in the presence of petrol. Also the interaction between the mixtures of diesel and kerosene (ratio 1:1, 1:3) with reference to diesel is above 1 but well below 2 indicating slight synergism whereas with reference to kerosene it is well above 2 indicating a higher level of synergism. From the foregoing diesel again is more toxic than kerosene contributing most to the toxicity of the mixture of diesel and kerosene. Consequently the toxicity of kerosene is enhanced in the presence of diesel. For the mixture of petrol and kerosene ratio 1:1 which is additive with reference to petrol, the petrol is more or less the sole cause of death to the test organisms. This implies that safe limits for refined petroleum products, which have been set on the basis of single action median lethal limits will likely fail to protect same set of sensitive species under condition where such toxicants occur in a mixture with other compounds that significantly synergise the action of the initial compound.

Furthermore, the results obtained in this study therefore underscores the need for joint action evaluations embracing the priority pollutants known to occur in contaminated ecosystems, when settling environmental safe limits of pollutants or verifies the ecological relevance of existing water criteria meant to achieve effective environmental protection of aquatic biota.

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