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Toxicity of mixtures of perfluorooctane sulphonic acid with chlorinated chemicals and lipid regulators

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ABSTRACT

The toxicological interaction of perfluorooctane sulphonic acid (PFOS) with the chlorinated pollutants triclosan and 2,4,6-trichlorophenol and the lipid regulators gemfibrozil and bezafibrate was evaluated using the combination index-isobologram equation. The endpoint for bioassays was the growth rate inhibition of the green alga *Pseudokirchneriella subcapitata*. The results showed that most of the binary combinations assayed exhibited antagonism at all effect levels. The addition of a third component induced a less antagonistic or even synergistic behaviour. This was particularly marked for the ternary mixture of triclosan and 2,4,6-trichlorophenol with PFOS, for which synergism was very strong at all effect levels, with a combination index as low as 0.034 ± 0.002 at EC_{50} for the mixture. The results obtained indicate that the evaluation of mixture toxicity from single component data using the concentration addition approach could severely underestimate combined toxicity.

1. Introduction

Perfluorooctane sulphonate and related compounds belong to the family of organic fluorinated chemicals in which carbon–hydrogen bonds are replaced by carbon fluorine bonds. These products have been used for decades for a variety of purposes, such as refrigerants, surfactants, water repellents, anticorrosion agents, and lubricants and waxes (Kissa, 2001; Mabury et al., 2002). The perfluorinated surfactants perfluorooctane sulphonic acid (PFOS) and perfluorooctanoic acid (PFOA) have been extensively detected in the environment and are of great environmental concern due to their persistence, bioaccumulation and biomagnification (Lehmler, 2005). Although PFOS was banned in Europe in 2006 and the major global producer of perfluorooctanesulfonyl fluoride (PFOS precursor) ceased production, their presence in the environment is repeatedly reported and this fact is a source of major concern to several protection agencies (EFSA, 2008; EPA, 2009; EEA, 2010). PFOS has been detected in surface water (Ahrens et al., 2010; Möller et al., 2010) and wildlife at numerous locations (Kannan et al., 2002; Martin et al., 2004). It has also been detected in drinking water and groundwater (Harada et al., 2003; Wilhelm et al., 2010). It has been clearly demonstrated that conventional waste water treatment plants (WWTP) are highly ineffective in treating several families of micropollutants, including perfluorochemicals, thereby contributing to significant pollution of receiving surface waters (Lin et al., 2010). Concern about the occurrence of PFOS has led to the publication of many toxicological studies on different organisms (Boudreau et al., 2003; Hanson et al., 2005; Bilbao et al., 2010).

Triclosan (TRI), 5-chloro-2-(2,4-dichlorophenoxy)-phenol, is an emergent pollutant widely used in consumer and professional health care products, such as disinfectant (Singer et al., 2002). From the early detection of Okumura and Nishikawa (1996) to the recent work of Rosal et al. (2010a), who reported an average concentration of 219 ng L^{-1} in the effluent of an activated WWTP, several studies have revealed that TRI is one of the most frequently detected compounds in surface water and wastewater. Binelli et al. (2009) associated TRI with genotoxic and cytotoxic effects in Zebra mussel hemocytes due to oxidative stress and possible direct DNA damage. TRI has been shown to be toxic to other aquatic organisms, such as *Daphnia magna*, with a 48-h EC_{50} of 390 μg L^{-1}, and the fish *Pimephales promelas*, with a 96-h lethal concentration of 260 μg L^{-1} (Orvos et al., 2002). Recently, An et al. (2009) measured the biochemical responses of *Triticum aestivum L*, demonstrating that the activity of peroxidise and superoxide dismutases in leaves and roots decreased with an increase in the concentration of TRI and exposure time. 2,4,6-trichlorophenol (TCP) has been used worldwide as an antimicrobial agent and wood preservative and even though its use has been prohibited or restricted after the discovery of its persistence, it has been repeatedly reported in environmental samples (Czaplicka, 2004). TCP, as well as other chlorophenols, can also originate in the disinfection of water with chlorine from chlorinated compounds and therefore chlorophenols

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can be found at relatively high concentrations in the environment (Ramamoorthy, 1997). It produces adverse effects on human health, fishes and benthic organisms (Meriläinen and Oikari, 2008), and has been classified by the USEPA as a priority pollutant.

Numerous studies have been conducted on the occurrence of pharmaceuticals in both WWTP effluents and receiving waters (Rosal et al., 2010a). Murray et al. (2010) reviewed the literature concerning the occurrence and toxicity of emerging pollutants and concluded that the highest priority pollutants for regulation should include not only industrial chemicals (PFOA, PFOS) and pesticides, but also pharmaceuticals and personal care products. Despite the amount of analytical data available, surprisingly little has been published on the ecotoxicological effects of many of these compounds on the organisms living in receiving streams, particularly as regards the potential toxic effects of mixtures of these compounds (Schnell et al., 2009). A major cause for concern is that hazard characterisation based on the determination of No Observed Effect Concentration (NOEC) for individual substances does not provide a safe rule for mixtures (Rodea-Palomares et al., 2010). Most studies published on mixtures of aqueous pollutants have attempted to explain the observed results in terms of the concepts of independent action and concentration addition.

The aim of this study was to evaluate the toxicity to the green microalgae *Pseudokirchneriella subcapitata* of binary and ternary mixtures of PFOS with chlorinated pollutants, namely TRI and TCP, and with the lipid regulators bezafibrate (BZ) and gemfibrozil (GM). The type and level of interactions established for these combinations of pollutants were determined according to the combination index (CI)-isobologram equation method established by Chou and Talalay (1984) to model the effect of drug combinations in pharmacology, which does not depend on the mode of action of individual compounds.

2. Materials and methods

2.1. Chemicals and toxicity evaluation

Perfluorooctane sulphonate potassium salt (PFOS, CAS No. 2795-39-3, 98%) was obtained from Fluka. Triclosan (TRI, CAS No. 3380-34-5, purity > 97%), TCP (CAS No. 88062, purity 98%), gemfibrozil (GM, CAS No. 25812-30-0, purity + 99%) and bezafibrate (BZ, CAS No. 41859-67-0, purity + 98%) were purchased from Sigma Aldrich. Algal beads of *P. subcapitata*, dissolving matrix and growing media were purchased from MicroBioTest Inc. (Belgium). The determination of multigenerational exposure toxicity was performed following the algal growth inhibition test described in OECD TG 201 *P. subcapitata* open system. The de-immobilization of algal cells was conducted according to the manufacturer's recommendations. Algal cells were first cultured in 25 mL shaken flasks in which growth was assessed by following optical density at 670 nm. The prescribed amount of cells was then transferred to 96-well clear disposable microplates and was exposed to pollutants during the logarithmic growth phase. The total volume occupied was 200 μL, which represents an important miniaturisation of the assay. *P. subcapitata* growth was monitored for 72 h, following optical density recorded by a Rayto RT-2100C microplate reader. Plates were incubated in a growing chamber at 22 ± 2 °C under continuous light. Each concentration was replicated four times in three independent series of assays. The stock solutions of TRI, TCP, GM, BZ and PFOS were prepared in pure water at concentrations near their corresponding solubility limits without addition of organic solvents. Serial dilutions of each compound, individually and with a fixed constant ratio (1:1) based on their individual EC_{50} values, were prepared using a dilution factor of 2 as follows: TRI + PFOS, TCP + PFOS, TRI + TCP, GM + PFOS, BZ + PFOS, GM + BZ. The ternary combinations TRI + TCP + PFOS and GM + BZ + PFOS were prepared following the same procedure. In accordance with the OCDE Guideline for Testing Chemicals (OECD, 2008), evaluation of photosensitive pollutant stability under chronic test conditions was performed using reversed-phase HPLC analysis at the start and at the end of the exposure period, as reported previously (Rosal et al., 2010b).

2.2. Calculation of the combination index (CI)

The results were analysed using the median effect/combination index (CI)-isobologram equation as reported by Chou and Talalay (1984) and Chou (2006). The computation is based on the median-effect principle that assumes that there is a univocal relationship between dose and effect irrespective of the number of compounds and their mechanism of action. The median effect equation is as follows:

$$\frac{f_a}{1-f_a} = \left(\frac{D}{EC_{50}}\right)^m \quad (1)$$

D represents the dose that affects a fraction f_a. EC_{50} represents the dose at which a 50% effect (growth inhibition) is reached. The exponent m accounts for the sigmoidicity of the dose–effect curve, where $m = 1$ for hyperbolic, $m > 1$ for sigmoidal and $m < 1$ for negative sigmoidal dose–effect curves. The EC_{50} (potency) and m (shape) values for each compound were determined by computing experimental f_a and D values. These parameters were then used to calculate the combined doses required to produce a given effect. For all effect levels, the combination index (CI) values were calculated according to the general combination index equation for n-chemical combination at x-percentage inhibition (Chou, 2006):

$$^{n}\mathrm{CI}_x = \sum_{j=1}^{n}\left[D_j/(D_x)_j\right] = \sum_{j=1}^{n}\frac{(D_x)_{1-n}\left[\frac{[D]_j}{\sum_1^n[D]}\right]}{(D_m)_j\left[\frac{(f_{ax})_j}{1-(f_{ax})_j}\right]^{\frac{1}{m_j}}} = \quad (2)$$

where $^{n}(\mathrm{CI})_x$ is the combination index for n chemicals at x% effect, $(D_x)_{1-n}$ is the sum of the dose of n chemicals that exerts x% inhibition in combination, $[D_j]/\sum_1^n[D]$ is the proportion of the dose of each chemical that exerts x% inhibition in combination and $(D_m)_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the dose of each drug alone that exerts x% inhibition. In the preceding equation, CI < 1, CI = 1 and CI > 1 indicates synergism, additive effect and antagonism, which were computed and represented using polygonograms.

3. Experimental results and discussion

Table 1 summarises the toxicity values obtained for the compounds assayed either individually or in binary and ternary mixtures. The toxicity to *P. subcapitata* of individual pollutants is expressed as an EC_{50} value (mg L^{-1}), with 95% confidence intervals (CI 95%) determined using a linear interpolation method independent of any particular dose–effect model (USEPA, 2002).

The results obtained for PFOS toxicity are in agreement with the values recently reported by Rosal et al. (2010c) and Boudreau et al. (2003) who obtained EC_{50} values for PFOS in the 48–88 mg L^{-1} for two algal species. Other non-ionic surfactants showed similar EC_{50} values for *P. subcapitata*, as those reported by Yamane et al. (1984). According to the obtained EC_{50} values, PFOS could be classified as harmful to algae. The toxicity of BZ and GM to several organisms, namely *Vibrio fischeri*, *D. magna* and the cyanobacteria *Anabaena* CPB4337, has been reported in a previous paper (Rosal et al., 2010b). Both compounds exhibited low toxicity to *P. subcapitata*, in line with data reported using other test species (Isidori et al., 2007). TCP and TRI were very toxic for the microalgae, with median

Table 1
Toxicity to *P. subcapitata* of individual pollutants and mixtures expressed as EC_{50} values (mg L^{-1}), dose effect parameters and mean combination index (CI) indicating 95% confidence intervals. EC_{10}, EC_{50} and EC_{90}, are the doses that inhibited growth by 10%, 50% and 90%, respectively. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect and antagonism, respectively. Synergistic responses have been stressed in bold.

Individual compounds	EC_{50}	Confidence interval (95%)
PFOS	35.0	34.2–35.5
Bezafibrate (BZ)	103.0	100.8–104.4
Gemfibrozil (GM)	48.8	48.4–49.2
Triclosan (TRI)	0.037	0.036–0.038
2,4,6-Trichlorophenol (TCP)	0.061	0.058–0.062

Mixtures	Dose effect parameters			CI values		
	EC_{50} (mg L^{-1})	m	r	EC_{10}	EC_{50}	EC_{90}
PFOS + TRI	41.1	1.18	0.991	4.16 ± 0.62	2.95 ± 0.18	2.38 ± 0.28
PFOS + TCP	12.2	0.98	0.994	**0.43 ± 0.08**	**0.65 ± 0.05**	1.00 ± 0.10
TRI + TCP	0.10	2.36	0.981	10.5 ± 1.6	2.76 ± 0.20	**0.73 ± 0.15**
PFOS + TRI + TCP	0.34	1.65	0.993	0.075 ± 0.009	**0.034 ± 0.002**	**0.017 ± 0.002**
PFOS + BZ	634.4	1.58	0.999	22.6 ± 7.1	10.4 ± 0.7	5.53 ± 0.68
PFOS + GM	187.7	0.983	0.965	3.07 ± 0.65	5.25 ± 0.54	9.28 ± 5.17
BZ + GM	654.3	1.04	0.992	11.3 ± 4.0	9.40 ± 1.16	8.49 ± 2.30
PFOS + BZ + GM	168.4	1.13	0.994	3.47 ± 0.85	2.92 ± 0.18	2.80 ± 0.47

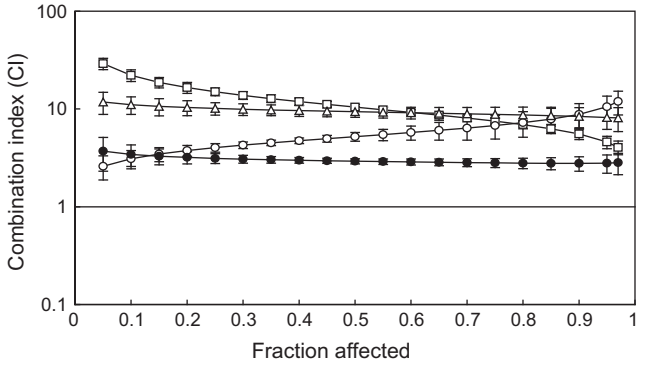

Fig. 1. Combination index plot for binary combinations containing fibrates: PFOS + BF (□), PFOS + GM (○), BF + GM (△) and the ternary mixture PFOS + BZ + GM (•). CI values are plotted as a function of the fraction of algal population affected by the toxicants (f_a). CI < 1 = 1 and >1 indicates synergism, additive effect and antagonism, respectively.

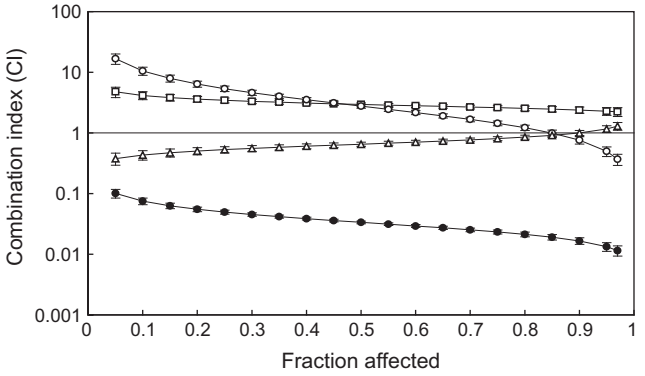

Fig. 2. Combination index plot for binary combinations containing chlorinated pollutants: PFOS + TRI (□), TRI + TCP (○), PFOS + TCP (△) and the ternary mixture PFOS + TRI + TCP (•). CI values are plotted as a function of the fraction of algal population affected by the toxicants (f_a). CI < 1 = 1 and >1 indicates synergism, additive effect and antagonism, respectively.

effect values in agreement with other previously published data (Orvos et al., 2002; Tatarazako et al., 2004; de Lorenzo et al., 2008; Rosal et al. 2010c).

Table 1 also shows the dose–effect relationship parameters and mean CI values for binary and ternary combinations. The parameters EC_{50}, m and r are the antilog of x-intercept, the slope and the linear correlation coefficient of the median-effect plot (Chou, 2006). CI values are reported at EC_{10}, EC_{50} and EC_{90}, respectively, representing the doses required to reach a growth inhibition of 10%, 50% and 90%. Synergism (CI < 1) has been emphasised in bold. The computer program CompuSyn (Chou and Martin, 2005) was used to calculate dose–effect curve parameters and CI values for different fractions affected f_a and to derive the polygonal graphic representations (polygonograms) depicting synergism, additive effect and antagonism. The EC_{50} values calculated for mixtures were also consistent with the assumption underlying the median-effect principle, as indicated by the linear correlation parameter, r, higher than 0.965 for all mixtures. In accordance with Chou and Martin (2005), the data from single pollutants and their mixtures were used to quantify the level of observed effect. The results for *P. subcapitata* as a function of the fraction affected are displayed in Figs. 1 and 2.

The data in Fig. 1 show that both binary and ternary mixtures containing fibrates exhibit antagonistic behaviour on algal growth independently of the affected fraction. CI values for the PFOS + BZ mixture varied from 22 ± 7 (very strong antagonism) to 5.5 ± 0.7 (strong antagonism) as effect level increased. For the binary mixture PFOS + GM, the pattern was the opposite, with CI changing from 3.1 ± 0.7 (antagonism) to 9 ± 5, and showing less marked variation with affected fraction. The mixtures of two fibrates (BZ + GM) also exhibited a similar degree of antagonism throughout the exposure range. The ternary mixture PFOS + BZ + GM presented a lower antagonism than the binary mixtures of the same compounds over the entire effect level range, with CI values essentially constant.

In a previous paper, Rodea-Palomares et al. (2010) studied the toxicological interaction of lipids regulators, namely gemfibrozil, bezafibrate and fenofibric acid, using the combination index-isobologram equation. This study was performed using two aquatic bioluminescent microorganisms, the marine bacterium *V. fischeri* and a recombinant cyanobacterium of the genus *Anabaena*. For the marine bacterium, the predominant interaction was antagonistic at low f_a levels, shifting to synergism at higher effect levels. *Anabaena* CPB4337 showed both synergism and antagonism effects, the former predominating at f_a levels below 0.4–0.5 and the latter for higher values. For Anabaena, ternary mixtures were generally more synergistically toxic than their corresponding

binaries, a result similar to that observed here for *P. subcapitata*. The main difference revealed by comparison of the same compounds assayed following the same experimental design but using different bioindicators, was the prevalence of antagonism at all affected fractions. The results presented in Table 1 for EC_{50} of the individual compounds indicate that the toxicological effect of fibrates on algal growth could be considerably reduced in the presence of PFOS, in both binary and ternary combinations with the other fibrate.

When triclosan was mixed with TCP, a very strong antagonistic effect was observed at low effect levels, changing to additivity at higher affected fractions. In a previous paper, we studied the toxicity of mixtures of TRI, TCP and docusate sodium, a surfactant showing similar toxicity to *P. subcapitata* as PFOS (Rosal et al., 2010c). Surprisingly, the binary combinations of docusate sodium with chlorinated compounds did not significantly change the EC_{50} of the surfactant alone. In this study, the EC_{50} of PFOS changes in binary and ternary combinations with the addition of chlorinated organics. The EC_{50} value of PFOS increased slightly in the presence of triclosan, but was lower for the mixture with TCP. A synergistic effect could be observed for PFOS + TCP for practically all *fa* levels. The binary mixture PFOS + TRI was antagonistic for algal growth, CI being practically unaltered for the whole range of affected fractions. In a previous study, Liu et al. (2009) evaluated the effect of PFOS on the toxicity of diuron, atrazine and pentachlorophenol to the green alga *Scenedesmus obliquus*. They concluded that PFOS affected the toxicity of organic compounds in different ways, probably modifying cell uptake. Antagonism was observed for diuron and atrazine and synergism for pentachlorophenol. All compounds mixed with PFOS were very toxic for *S. obliquus*, but the degree of interaction was not quantified. The ternary mixture of chlorinated pollutants with PFOS showed very strong synergism for all effect levels (CI < 0.1). This ternary combination was particularly relevant as an indication of how the type of toxicological interaction may change in ternary mixtures with respect to their binaries. In a previous study by Rosal et al. (2010c), a similar result was observed for the ternary mixture of the same chlorinated compounds with the surfactant docusate sodium; however, the combination with docusate was synergistic at f_a levels above 0.6, in contrast to the results obtained with PFOS, where synergism was extreme (Fig. 2).

The comparison of the toxicological patterns developed in *P subcapitata* by the mixtures studied revealed some similarities. The presence of PFOS modifies the toxicity of all compounds, regardless of the level of toxicity exerted by individual pollutants. Accordingly, the CI–f_a curves shown in Figs. 1 and 2 for ternary combinations always appear below those for binary mixtures, suggesting that the co-existence of other pollutants with PFOS represents an enhanced risk to aquatic ecosystems. The ternary mixture including chlorinated compounds constitutes a particularly hazardous combination for algal growth. The effect of binary mixtures can be plotted as polygonograms (Chou, 2006). Fig. 3 shows the polygonograms at two effect levels for all the mixtures, providing a simplified depiction of the overall results for the type and level of effects observed for each combination. Synergism is indicated by solid lines and antagonism by broken ones; the thickness of the lines indicates the strength of the interaction. These representations show how antagonism is generalised, with the exception of the binary mixture TRI–TCP.

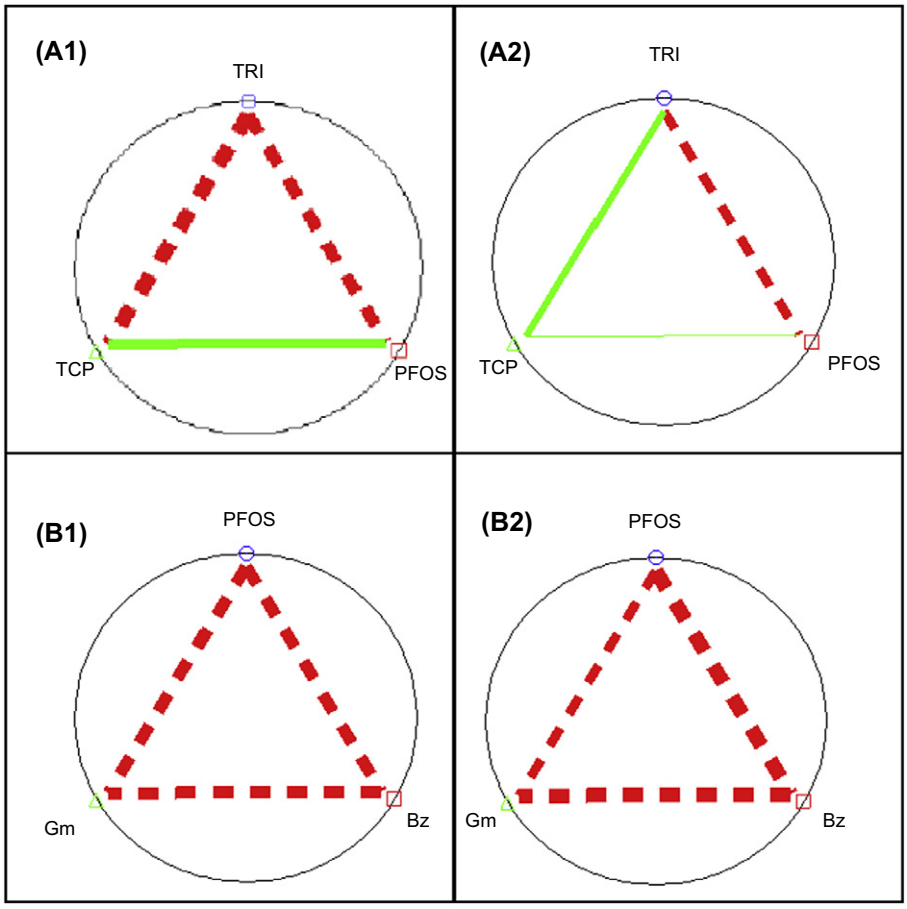

Fig. 3. Polygonograms showing the toxicological interactions in binary combinations of pollutants at two effect levels. Chlorinated compounds and PFOS: (A1) for f_a = 0.1: (A2) for f_a = 0.9. Fibrates and PFOS: (B1) at f_a = 0.1; (B2). at f_a = 0.9.

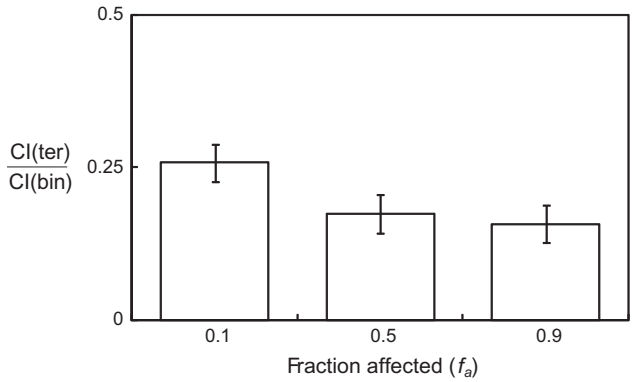

Fig. 4. Average combination index for ternary mixtures relative to their respective binaries at different effect levels. The bars represent standard error.

Fig. 4 shows the value of CI for ternary mixtures relative to the CI of the three binary mixtures formed in every ternary combination. The plot shows the average for all data reported in this study as well as those from mixtures with docusate sodium published elsewhere (Rosal et al., 2010c). It is clear that all values are below unity, indicating that all ternary mixtures were systematically more synergistic than their binaries to a factor of at least four. This affirmation is in accordance with a previously published work (Rodea-Palomares et al., 2010), in which it was study a very complex mixture including pharmaceuticals and a real wastewater from a local treatment plant, which is a mixture of thirty micropollutants. In this work, it was reported that synergism was the predominant interaction observed in presence of wastewater in a wide range of effect level for *Anabaena* CPB4337. Koutsaftis and Aoyama (2007) examined the toxicity of binary, ternary and quaternary combination of antifouling biocides on the brine shrimp *Artemia salina* and found that the complexity of the mixture tends to increase the relevance of synergistic effects. These results agree with our previous research, which showed that the nature of the toxicological interaction depends on the effect level of the mixture, but is less influenced by the toxicological mode of action of each component. It is interesting to point out that classic response addition or Bliss independence, usually considered a particular case of the absence of interaction for the case of mutually non-exclusive drugs, receives a different treatment in the CI-isobologram approach followed in this work. Chou and Talalay (1984) and Chou (2006) defined additivity only for the case of mutually exclusive drugs, which is considered the universal standard for additivity in drug interaction analysis. Bliss independence is considered a particular case of synergism, so that when dealing with mixtures of compounds with different mode of toxic action, one should expect a synergistic CI-isobologram response.

4. Conclusions

The application of the combination index (CI)-isobologram equation to mixtures of PFOS with the chlorinated pollutants TRI and TCP and the fibrates GM and BZ revealed that most binary mixtures behaved antagonistically towards growth inhibition of the green alga *P. subcapitata*. This behaviour became less antagonistic or even synergistic in ternary mixtures. The increased toxicity of the ternary mixtures was particularly marked for the ternary mixture of chlorinated pollutants (TRI + TCP) with PFOS, which showed a very strong synergism at all effect levels. This ternary combination demonstrates how the type of toxicological interaction may change in ternary mixtures with respect to their binaries. These results suggest that the risk associated with the co-occurrence of this surfactant with other xenobiotics may be severely underestimated by additive approaches. The displacement from antagonistic to synergistic behaviour in complex mixtures with only three components may indicate that the evaluation of mixture toxicity from single component data assuming additive behaviour requires revision. It should be noted that the CI method does not require any previous knowledge of the mode of toxic action of chemicals.

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