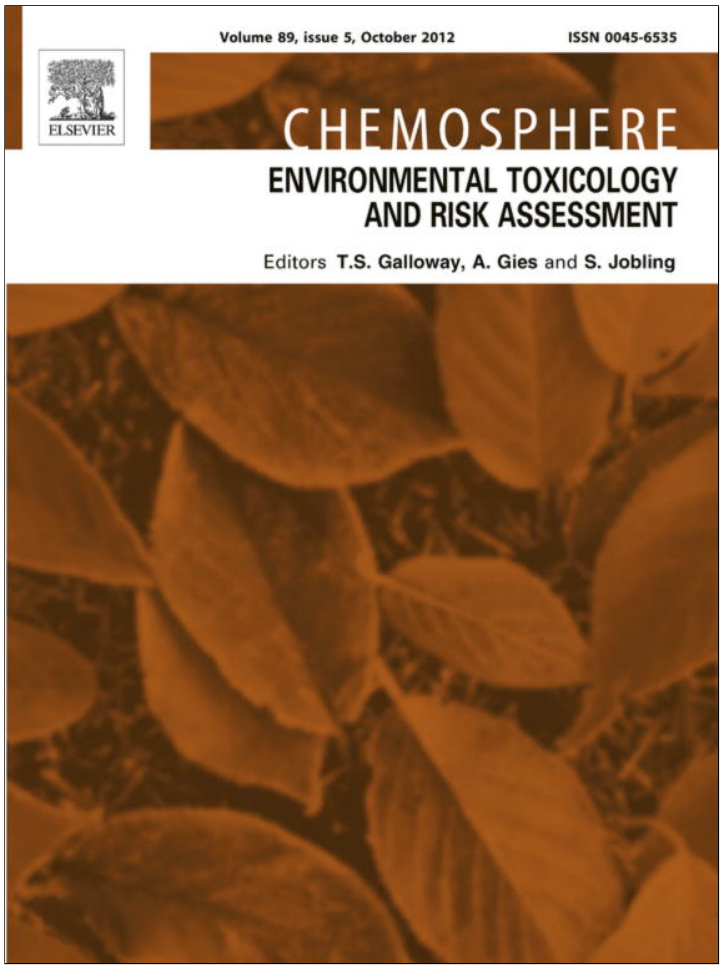

Chemosphere 89 (2012) 592–601

Contents lists available at SciVerse ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Effects of the aquatic contaminant human pharmaceuticals and their mixtures on the proliferation and migratory responses of the bioindicator freshwater ciliate Tetrahymena

Júlia Láng, László Kőhidai *

Department of Genetics, Cell- and Immunobiology, Semmelweis University, PO Box 370, H-1445 Budapest, Hungary

HIGHLIGHTS

- Acute toxic effects of NSAIDs, β-blockers are unlikely to *T. pyriformis*.
- Antibiotics and Na-diatrizoate do not inhibit Tetrahymena proliferation.
- Chemotaxis is a more sensitive cell physiological response than proliferation.
- In binary pharmaceutical mixtures, the interaction types are concentration dependent.

ARTICLE INFO

Article history:
Received 2 December 2011
Received in revised form 23 April 2012
Accepted 16 May 2012
Available online 12 June 2012

Keywords:
Toxicity
Chemotaxis
Mixture
Pharmaceutical
Tetrahymena
NSAID

ABSTRACT

An increasing attention is paid to the potential harmful effects of aquatic contaminant pharmaceuticals exerted on both biosystems and humans. In the present work the effects of 14 pharmaceuticals including NSAIDs, antibiotics, β-blockers and a frequently used X-ray contrast media on the proliferation and migratory behavior of the freshwater ciliate *Tetrahymena pyriformis* was investigated. Moreover, the mixture toxicity of four selected pharmaceuticals (diclofenac, ibuprofen, metoprolol and propranolol) was evaluated in binary mixtures using full factorial experimental design. Our results showed that the sensitivity of Tetrahymena to NSAIDs and β-blockers (EC_{50} ranged from 4.8 mg L^{-1} to 308.1 mg L^{-1}) was comparable to that of algal or Daphnia bioassays. Based on these elevated EC_{50} values acute toxic effects of these pharmaceuticals to *T. pyriformis* are unlikely. Antibiotics and the contrast agent sodium-diatrizoate had no proliferation inhibiting effect. Chemotactic response of Tetrahymena was more sensible than proliferation as significant chemorepellent action was observed in the environmentally realistic concentration range for acetylsalicylic acid, diclofenac, fenoprofen, paracetamol, metoprolol, propranolol, timolol and trimethoprim (Chemotaxis Index ranged from 63% to 88%).

Mixture toxicity experiments resulted in a complex, concentration dependent interaction type pattern with antagonism being the predominant interaction type (59%) followed by additivity (37%) and synergism (4%). Hence the concept of concentration addition validated for NSAIDs in other organisms cannot be adopted for this ciliate.

In summary authors suggest Tetrahymena as a sensible model of testing aquatic contaminants as well as underline the significance using more specific endpoints to understand the complex mechanisms investigated.

1. Introduction

The exponentially increasing number of scientific publications dealing with the occurrence of human and veterinary pharmaceuticals in the aquatic environment settles all doubts about the ubiquitous presence of these chemicals in water streams. They mainly enter the aquatic environment by municipal wastewater where they are disposed or excreted to following metabolization. Some of these compounds resist to conventional wastewater treatment technologies or they may be transformed into toxic products and end up in surface waters receiving the contaminated wastewater effluents. Aquatic ecosystems are important targets of pollutant pharmaceuticals as these organisms are exposed to these compounds over their whole life via wastewater residues (Fent et al., 2006). More over, pharmaceuticals are designed to have specific mode of action and many of them for persistence in the organism.

* Corresponding author. Tel.: +36 1 2102930; fax: +36 1 3036968.
E-mail address: kohlasz2@gmail.com (L. Kőhidai).

0045-6535/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.chemosphere.2012.05.058

The occurrence pattern and the concentration of the wide range of pharmaceutically active compounds largely vary from country to country. Nevertheless, the most frequently detected prescription classes include non-steroidal antiinflammatory drugs (NSAIDs), antibiotics, beta-adrenergic antagonists (β-blockers) and iodinated X-ray contrast media. The observed levels of these aquatic contaminants (Table 1) that range from the low ng L^{-1}–μg L^{-1} are prognosticated to increase as the consumption of these drugs is predicted to rise further (Fent et al., 2006).

The environmental risk assessment of these compounds is encumbered by several difficulties such as the lack of ecotoxicity data especially chronic ones and measuring tools that are able to detect and monitor the presence of pharmaceuticals in surface waters. Nevertheless the range of the available ecotoxicity data is often overlapping with the detected environmental concentrations as in the case of diclofenac, ibuprofen, paracetamol and sulfamethoxazole especially if these compounds are present in mixtures (Pal et al., 2010).

The complex exposure situations caused by the aquatic pollutants can be in general realistically described by the concept of concentration addition as most of the contaminants are nonpolar organic compounds that show no specific mode of action and whose toxicity is governed by hydrophobicity (Fent et al., 2006). This concept implies that substances applied at low or no effect concentration can contribute to the total mixture effect which in turn can become significant. Concentration addition was observed e.g. for NSAIDs in Daphnia and algal bioassays by Cleuvers (2003).*Tetrahymena pyriformis* is a non-pathogenic freshwater ciliate protozoan the abundance of which may indicate healthy aquatic environments. It represents an important trophic level where bioaccumulation processes may be significant (Gerhardt et al., 2010). As a microbial fauna member at WWTPs, this species was found to ameliorate the stability and performance of biological wastewater treatment (Gerhardt et al., 2010). Besides practical advantages such as short generation time (~150 min) which allows a relatively large number of generations (~10) to be studied even during 24 h of exposure, the use of Tetrahymena may also be favorable because it shows a lot of similarities with higher ranked vertebrates in terms of e.g. receptors (Kőhidai and Csaba, 1995) and second messenger systems (Csaba and Németh, 1980). Tetrahymena growth impairment tests were reported to show significantly higher sensitivity to diverse xenobiotics (e.g. heavy metals, insecticides) compared to bacterial tests such as MicroTox test (Bogaerts et al., 2001) or to a human hepatoma cell line based cytotoxicity assay (Rudzok et al., 2011). A direct comparison of the change in viability by Tetrahymena and fish cells in response to heavy metals showed some very similar responses as well as some differences (Dayeh et al., 2005). The interspecies correlation study of Zhang et al. (2010) showed a poor interspecies correlation between *T. pyriformis*, algae and *D. magna* for organic chemicals which suggests a high level diversity in the uptake and toxic mechanisms and underlines the importance of the simultaneous use of different species in ecotoxicity assessments (Zhang et al., 2010). Beside growth impairment tests, Tetrahymena behavioral assays studying migratory responses of this motile organism are also common. A very broad range of biologically active natural compounds e.g. hormones (Csaba and Németh, 1980) or anthropogenic chemicals including pentachlorophenol, opiod drugs and chloramphenicol (Sauvant et al., 1999) were described to influence Tetrahymena migratory behavior in sublethal, nanomolar concentrations. On the basis of the above described advantages of the Tetrahymena model, in the present work our aim was

(i) to screen the toxicity of fourteen human pharmaceuticals (Table 1) that belong to the most frequently detected prescription classes (anti-inflammatory and analgesic drugs, antibiotics, β-blockers and X-ray contrast media) to the *T. pyriformis*;
(ii) to describe the type of interactions of the most toxic pharmaceuticals in combinations (additivity, synergism or antagonism);
(iii) to study the influence of sublethal, ecotoxicologically relevant concentrations of drugs on the chemotactic responses of the model organism.

2. Materials and methods

2.1. Cell culturing

Tetrahymena pyriformis GL cells were maintained in 0.1% yeast extract containing 1% Bacto tryptone (Difco, Michigan, USA) medium at 28 °C. For both the chemotaxis and the growth inhibition assays 24 h old, exponential growth phase cultures were used.

Table 1
Mechanism of action, logarithmized partitioning coefficient ($\log K_{ow}$) and environmental concentrations of the pharmaceuticals tested.

Pharmaceutical	$\log K_{ow}$ *	Action (Fürst, 2004)	Environmental concentration (ng L^{-1})	
			WWTP effluent	Surface water
Acetylsalicylic acid	1.2	NSAID; nonselective inhibition of COX-2**	150 (Fent et al., 2006)	200 (Fent et al., 2006)
Diclofenac	4.4		460–3300 (Pal et al., 2010)	21–41 (Pal et al., 2010)
Fenoprofen	3.3		300 (Fent et al., 2006)	80–120 (Fent et al., 2006)
Ibuprofen	3.5		134–7100 (Pal et al., 2010)	14–44 (Pal et al., 2010)
Naproxen	3.3		450–1840 (Pal et al., 2010)	<0.3–146 (Pal et al., 2010)
Paracetamol	0.5	Analgesic-antipyretic; inhibition of COX-3?	59–220 (Pal et al., 2010)	12–777 (Pal et al., 2010)
Erythromycin	2.7	Bacteriostatic; inhibition of the 50S ribosome subunit during translation	<287 (Kümmerer, 2009)	4.5 (Zuccato et al., 2005)
Lincomycin	0.2		24.4 (Zuccato et al., 2005)	<730 (Kümmerer, 2009)
Sulfamethoxazole	0.9	Bacteriostatic; inhibition of the bacterial dihydropteroate syntethase involved in DNA replication	91–794 (Pal et al., 2010)	<0.5–4 (Pal et al., 2010)
Trimethoprim	0.9	Bacteriostatic; inhibition of the bacterial dihydrofolate reductase involved in DNA replication	99–1264 (Pal et al., 2010)	0–78.2 (Pal et al., 2010)
Metoprolol	1.9	Blocking of the β-adrenergic receptor	15–900 (Fent et al., 2006)	30 (Fent et al., 2006)
Propranolol	3.0		30–44 (Pal et al., 2010)	20 (Pal et al., 2010)
Timolol	1.8		5.5 (Gabet-Giraud et al., 2010)	No data
Na-diatrizoate	1.8	X – ray contrast agent	80 (Ternes, 2001)	2 (Putschew et al., 2000)

* estimated partitioning coefficients of the non-ionized compounds, retrieved from the database of PubChem project (http://pubchem.ncbi.nlm.nih.gov/).
** COX: Cyclooxygenase.

2.2. Pharmaceuticals

In both chemotaxis and single compound toxicity assays 14 human pharmaceuticals were tested including five NSAIDs, one antipyretic–analgesic drug, four antibiotics, three β-blockers and an X-ray contrast agent (Table 1). All compounds were obtained from Sigma Aldrich, Germany. Water soluble drugs (diclofenac-Na, ibuprofen-Na, naproxen-Na, lincomycin hydrochloride, metoprolol-tartarate, propranolol-hydrochloride, timolol maleate, Na-diatrizoate) were diluted in the culture media; whereas hydrophobic substances (acetylsalicylic acid, fenoprofen-Ca, paracetamol, erythromycin, sulfamethoxazole, trimethoprim) were diluted in DMSO. The concentration of stock solutions was uniformly 0.05 M, the consecutive dilutions of the stock solutions were made in culture media. For the sake of simplicity pharmaceuticals will be referred hereinafter as the name of the active agent.

2.3. Growth inhibition assay of single pharmaceuticals and mixtures

For all toxicity tests 96-well plates (Sarstedt, Germany) were seeded with 10^3 cells well^{-1}. Then the cells were exposed to single pharmaceuticals or binary combinations of them for 24 h. For each measurement points at least 18 replicas were done. Following fixation with PBS containing 4% formaldehyde the cell number was determined using the CASY® TT cell counter system (Innovatis-Roche, Switzerland). The function of the equipment is based on the electric current exclusion principle. The cell number and size are registered based on the number and amplitude of these electrical signals, respectively. This means that when cells pass through a pair of electrode they increase resistance due to their insulating plasma membrane. For Tetrahymena the considered particle diameter ranged from 10 to 100 μm.

Growth inhibition (%) was calculated as the ratio of the average cell number in the sample wells and the average cell number in control wells. For hydrophilic drugs control wells contained pure culture media, whereas for hydrophobic ones culture media containing the adequate volume ratio of DMSO served as a control.

In the single compound growth inhibition assays that aimed to identify the most toxic pharmaceuticals the concentration range was tested in 10^{-11}–10^{-3} M using a 10-fold dilution between each measurement point. In the second step of the test the two most toxic hydrophilic members of the two most potent growth inhibitor prescription classes were selected to be tested in binary mixtures. For this purpose first their individual concentration-growth inhibition curves were established using concentration levels determined by preceding range-finding tests. For the details of the curve fitting see Section 2.5.

Binary mixtures were prepared from the four selected drugs in all the six possible combinations. The toxicity of these mixtures was studied using the concept of concentration addition (Berenbaum, 1985) and the Toxic Unit approach (Sprague, 1970) that expresses the concentration of each mixture constituent as the ratio of its actual concentration and its EC_{50} value. Concentrations of mixture constituents were set according to a 2^4 full factorial design in which individual factor levels corresponded to 0.25, 0.50, 0.75 and 1.0 Toxic Unit (TU).

2.4. Chemotaxis assay

The measurement of the chemotactic responses induced by the 14 pharmaceuticals took place in a two chamber multichannel capillary assay described by Kőhidai (1995) at 28 °C and at normal lighting conditions. For this purpose cells were placed in a 96-well plate (lower chamber) at 10^5 cells well^{-1} density whereas test substances were filled in the tips of a multichannel pipette (upper chambers) placed above the wells containing the cell suspension. The positive responder cells detect developing concentration gradients in the microscopic proximity of capillary openings and this advantageous chemical signal guides chemotactically active cells into the upper chamber, while negative responders migrate away. Our previous experiments has proved that taking short incubations times (5–20 min) for the assay facilitate to gain pure gradient directed chemotactic responses and prevent the contamination of the samples from randomly running chemokinetic responder cells (Schiess et al., 2001). Following 20 min of incubation positive responder cells were fixed and counted with the CASY® TT cell counter system. The tested drug concentration varied from 10^{-15} M to 10^{-6} M and each measurement was done in 12 replicas. Chemoattractant or repellent effect of pharmaceuticals was quantified by the '*Chemotaxis Index*' (Chtx. Ind.) which was calculated as the ratio of the number of cells that migrated towards the chamber containing the test substance and the number of cells that migrated towards the chamber containing the reference culture media as mentioned in Section 2.3.

2.5. Statistical analysis and curve fitting

In both chemotaxis assay and growth inhibition assay statistical significance of the results compared to the respective controls was calculated with one-way analysis of variance (ANOVA) in the Origin7.0. In mixture toxicity assay the obtained growth inhibition effects of mixtures were compared to the sum of the individual growth inhibition effects of the two mixture constituents and the significance of the difference was evaluated by one-way ANOVA. Interaction of two drugs was considered as a 'simple additivity' if the observed mixture toxicity did not differ significantly from the sum of the individual toxic effects. If the growth inhibition exerted by the mixture of the two components was significantly higher than the sum of their individual toxic effects it was described as 'synergism'. On the contrary, interaction was considered to be an 'antagonism' if the combined toxic effect was significantly lower than the sum of the individual toxicity values. In the case of the four selected pharmaceuticals (see Section 2.3) individual concentration-growth inhibition curves were established. Based on the assumption of a monocausal relationship between concentration and response, symmetric sigmoidal curves were fitted to measurement data (8 measurement points for each pharmaceutical with 18 replicas at each point) in the Origin7.0. For the sigmoidal curve fitting a four parameter logistic function was used by the following equation:

$$f(x) = \frac{A_1 - A_2}{1 + (\frac{x}{x_0})^p} + A_2 \quad (1)$$

where A_1 upper asymptote represents the maximal proliferation inhibition (about 100%); A_2 lower asymptote stands for the minimal proliferation inhibition (about 0%); x_0 represents the concentration at which proliferation inhibition equals 50% (the EC_{50} value) and p stands for the slope. The standard errors of the parameters obtained were also evaluated. For each pharmaceutical the individual toxic effects at the concentrations representing 25%, 50%, 75% and 100% of the respective EC_{50} values were determined using the fitted curves.

3. Results

3.1. Growth inhibiting effects of single pharmaceuticals

Toxicity screening of the 14 pharmaceuticals showed that NSAIDs and β-blockers were the most toxic classes to *T. pyriformis* as all of the five NSAIDs and three β-blockers exhibited significant growth inhibiting effect in the 10^{-11}–10^{-3} M range (Table 2).

Table 2
Concentration dependent impact on growth of the fourteen drugs tested in the first toxicity screening.

Substance	Effect on growth (%)*								
	10^{-11} M	10^{-10} M	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
Acetylsalicylic acid	**−17.2 ± 6.0^{x}**	**−16.6 ± 6.0^{x}**	**−25.0 ± 3.6^{y}**	**−26.0 ± 3.5^{z}**	−13.2 ± 7.8	−7.6 ± 3.2	−11.6 ± 4.2	+1.0 ± 4.7	+1.2 ± 4.2
Diclofenac	+24.6 ± 14.7	+4.4 ± 16.1	+14.4 ± 19.5	+26.5 ± 13.8	+30.0 ± 20.2	+8.8 ± 28.0	+0.5 ± 14.3	**−35.6 ± 8.2^{x}**	**−76.4 ± 5.1^{z}**
Fenoprofen	+10.8 ± 5.0	+15.0 ± 7.3	+3.0 ± 5.2	−12.0 ± 9.9	−6.2 ± 3.8	−1.0 ± 4.3	−4.8 ± 4.3	**−21.6 ± 3.6^{y}**	**−82.6 ± 3.2^{z}**
Ibuprofen	+32.0 ± 27.3	+35.6 ± 24.1	+16.0 ± 17.8	+19.8 ± 24.4	+14.6 ± 18.0	−4.0 ± 23.0	+5.0 ± 23.0	+23.2 ± 24.7	**−71.4 ± 3.7^{y}**
Naproxen	−5.4 ± 8.0	−4.6 ± 11.5	−21.3 ± 15.4	+0.4 ± 6.5	−7.8 ± 11.4	−1.0 ± 4.9	+2.2 ± 10.6	+6.8 ± 10.3	**−48.8 ± 8.7^{y}**
Paracetamol	+0.8 ± 11.8	+10.7 ± 12.5	+2.6 ± 11.3	+5.4 ± 17.8	+18.2 ± 13.9	+10.8 ± 12.1	+20.6 ± 17.9	+28.8 ± 15.3	**+42.0 ± 11.5^{x}**
Erythromycin	+11.2 ± 11.6	**+25.4 ± 5.6^{y}**	**+19.6 ± 3.4^{y}**	**+13.4 ± 4.9^{x}**	+7.4 ± 4.5	+2.6 ± 4.2	+10.4 ± 16.0	+0.6 ± 9.6	0.0 ± 10.7
Lincomycin	+13.9 ± 3.1	+11.6 ± 1.5	+0.3 ± 3.4	−10.3 ± 5.3	−1.4 ± 2.8	−0.1 ± 1.4	−5.3 ± 5.7	−5.2 ± 2.2	−7.1 ± 4.4
Sulfamethoxazole	−3.4 ± 5.9	+18.8 ± 9.4	+4.2 ± 4.9	−6.8 ± 4.9	−12.6 ± 4.3	−6.8 ± 4.6	−22.2 ± 5.5	+0.2 ± 5.8	−21.8 ± 2.9
Trimethoprim	−8.6 ± 6.2	+8.8 ± 7.2	−0.8 ± 5.7	−0.75 ± 5.7	**−24.6 ± 4.6^{y}**	**−20.0 ± 4.2^{y}**	**−11.6 ± 7.5^{x}**	**−15.0 ± 3.2^{x}**	**−69.0 ± 4.9^{y}**
Metoprolol	+3.1 ± 8.0	+9.7 ± 4.0	+2.7 ± 1.8	−0.4 ± 3.3	+3.4 ± 4.7	+17.1 ± 3.6	**−12.4 ± 1.9^{x}**	**−41.3 ± 6.6^{y}**	**−82.0 ± 2.4^{z}**
Propranolol	−10.2 ± 4.2	+27.0 ± 11.3	−0.6 ± 10.5	−8.8 ± 10.2	+2.6 ± 14.9	**−13.5 ± 3.5^{x}**	**−39.1 ± 11.9^{y}**	**−91.1 ± 1.9^{z}**	**−94.2 ± 0.8^{z}**
Timolol	−4.1 ± 7.34	+4.7 ± 3.72	+7.7 ± 2.7	+3.6 ± 2.7	+3.1 ± 2.5	−5.0 ± 4.7	−20.5 ± 11.7	**−29.9 ± 4.9^{y}**	**−75.9 ± 1.3^{z}**
Na-diatrizoate	+13.6 ± 2.6	**+15.1 ± 2.0^{x}**	+7.3 ± 14.3	+7.1 ± 14.4	+15.4 ± 5.0	+10.1 ± 2.8	+1.3 ± 8.0	−12.0 ± 12.1	**+16.0 ± 2.7^{x}**

* "+" values correspond to growth enhancement; "−" values represent growth inhibition. Significant effects are written in bold. Significance levels are x: $p < 0.05$; y: $p < 0.01$; z: $p < 0.001$.

However, except for acetyl salicylic acid these drugs inhibited cell proliferation only in the 10^{-5}–10^{-3} M concentrations (growth inhibition ranged from 12.4 ± 1.9% for 10^{-5} M ibuprofen to 94.2 ± 0.8% for propranolol at 10^{-3} M) which are at least three order of magnitude higher than the environmental concentrations. On the contrary, acetyl salicylic acid had significant but less pronounced effect (16.6 ± 6.0%–26.0 ± 3.5%) in the 10^{-11}–10^{-8} M range. Hence for NSAIDs the increasing order of toxicity based on the proliferation inhibition at the respective lowest effective concentrations was naproxen < ibuprofen < diclofenac < fenoprofen < acetyl salicylic acid. The antipyretic–analgesic paracetamol in turn significantly promoted cell proliferation with 42.0 ± 11.5% at 10^{-3} M and similar but weaker effect was also observed in the entire concentration range. From β-blockers propranolol proved to be the most potent as it showed 13.5 ± 3.5% growth inhibition at 10^{-6} M whereas significant growth inhibiting effect of metoprolol and timolol was observed above 10^{-5} M and 10^{-4} M respectively. For metoprolol growth inhibition increased from 12.4 ± 4.0% to 82.0 ± 2.4% with the rising concentrations. In the case of timolol growth inhibiting effects were 29.9 ± 4.9% at 10^{-4} M and 75.9 ± 1.3% at 10^{-3} M. Consequently the observed order of toxicity was timolol < metoprolol < propranolol.

Sulfamethoxazole and lincomycin did not affected the cell proliferation significantly at any concentration assayed, whereas erythromycin had proliferation promoting effect at 10^{-11}–10^{-5} M, particularly in the 10^{-10}–10^{-8} M range where proliferation was enhanced with 25.4 ± 5.6%, 19.6 ± 3.6% and 13.4 ± 4.9% respectively. The iodinated X-ray contrast agent Na-diatrizoate showed no proliferation inhibition at any concentration, on the contrary it had slight proliferation enhancing effect in the entire concentration range (that did not exceed 16.0 ± 2.7%).

Based on the results listed above diclofenac, ibuprofen, metoprolol and propranolol were chosen for the study of combined effects in binary mixtures. This selection was made using three criteria: (i) the most toxic, (ii) water soluble pharmaceuticals were selected, (iii) two compounds from two different prescription classes so that combined effects of pharmaceuticals with both similar and different mode of actions could be studied. As a first step individual concentration–growth inhibition curves were established for these compounds (Fig. 1). It was found that EC_{50} values were $8.35 \times 10^{-5} \pm 7.09 \times 10^{-6}$ M (26.56 ± 2.26 mg L^{-1}) for diclofenac, $2.05 \times 10^{-4} \pm 7.81 \times 10^{-6}$ M (46.79 ± 1.78 mg L^{-1}) for ibuprofen, $4.51 \times 10^{-4} \pm 2.30 \times 10^{-5}$ M (308.85 ± 15.75 mg L^{-1}) for metoprolol and $1.63 \times 10^{-5} \pm 9.45 \times 10^{-7}$ M (4.82 ± 0.28 mg L^{-1}) for propranolol. Growth inhibition effects corresponding to the 25%, 50%, 75% and 100% of the respective EC_{50} values were predicted base on the fitted curves and checked by growth inhibition assay. The difference of predicted and measured data was not significant in any case. Respective predicted values were 14%, 29%, 41% and 50% for diclofenac, whereas measured effects were 15.9 ± 5.7%, 30.4 ± 5.5%, 42.2 ± 3.7% and 48.3 ± 1.9%. In the case of ibuprofen predicted effect values were 10%, 25%, 39%, 50% and measured growth inhibitions were 14.6 ± 4.8%, 33.1 ± 8.1%, 41.9 ± 5.9% and 47.1 ± 3.1%. For metoprolol predicted data were 20%, 33%, 43% and 50%, on the other hand measurement data were 19.4 ± 10.1%, 35.3 ± 8.2%, 37.5 ± 9.8% and 49.9 ± 6.3%. Finally, for propranolol predicted growth inhibitions were 10%, 25%, 39% and 50% while measured data were 14.8 ± 8.5%, 23.1 ± 9.5%, 36.3 ± 13% and 46.8 ± 7.3%.

3.2. Toxicity of binary mixtures

Combined effects of diclofenac, ibuprofen, metoprolol and propranolol were studied in all of the six possible binary mixtures. Observed mixture proliferation inhibiting effects were compared to the sum of the individual proliferation inhibitions of the two mixture constituents at their respective actual concentrations in order to determine the type of interaction, i.e. additivity, synergism, antagonism (as described in Section 2.5).

Using the *diclofenac + ibuprofen* mixtures additivity occurred only in the mixture containing the lowest concentration of both diclofenac and ibuprofen (0.25 TU diclofenac + 0.25 TU ibuprofen) while at all other concentrations antagonism was the observed type of interaction (Appendix A, Table A1). In the *diclofenac + metoprolol* combinations additivity was the predominant form of combined action, synergism was observed in the case of the mixture 1 TU diclofenac + 0.25 TU metoprolol and with the three mixtures of 1.5 TU total concentration (0.5 TU diclofenac + 1 TU metoprolol; 0.75 TU diclofenac + 0.75 TU metoprolol; 1 TU diclofenac + 0.5 TU metoprolol) (Appendix A, Table A2). Antagonism was observed at the highest concentration (1 TU diclofenac + 1 TU metoprolol). Using the *diclofenac + propranolol* mixtures additivity was observed in seven cases and antagonism was observed in nine cases (Appendix A, Table A3). Interestingly, the two interaction types alternated. At the two lowest mixture concentrations (0.5 TU and 0.75 TU) additivity was observed whereas at intermediate mixture concentration (1 TU, 1.25 TU and 1.5 TU) both additivity and antagonism was observed. At the highest mixture concentrations (1.75 TU and 2 TU) the interaction type was antagonism. Similarly, with the *ibuprofen + metoprolol* mixtures at the lower mixtures

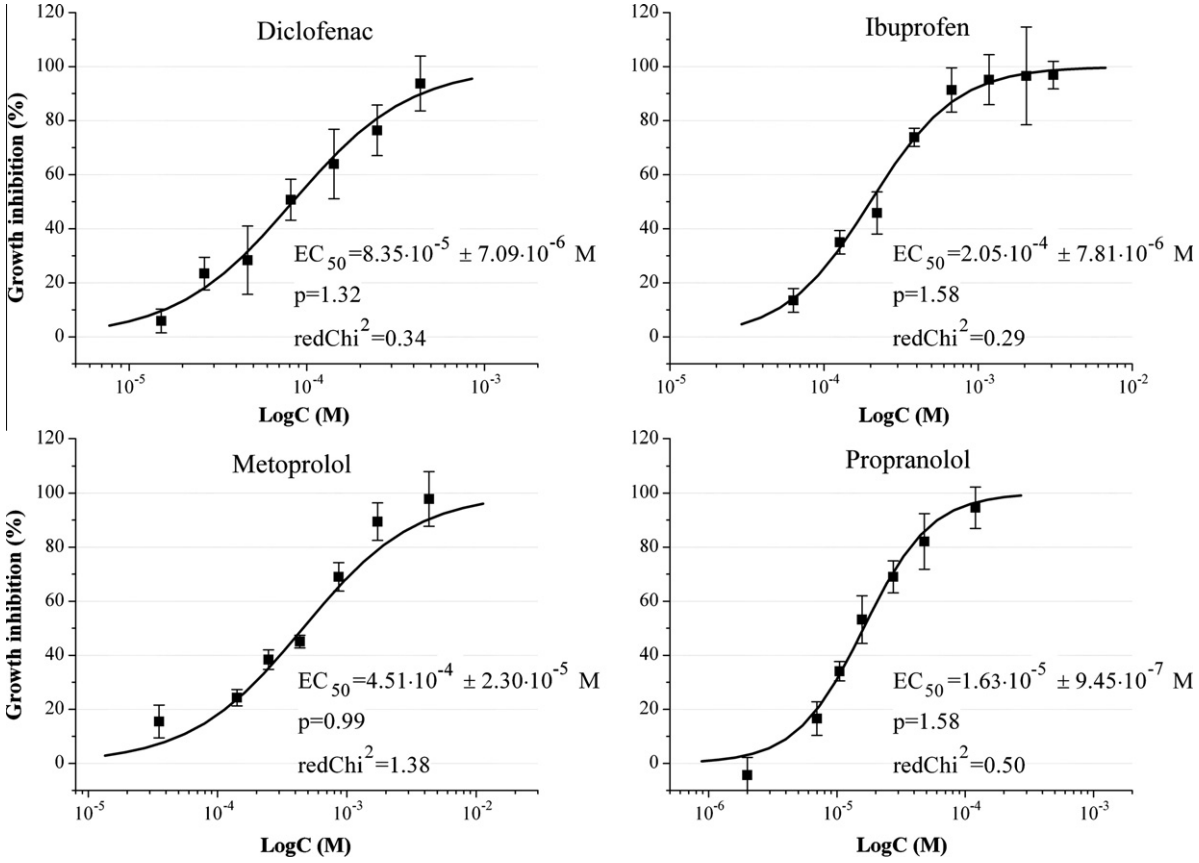

Fig. 1. Fitted concentration–growth inhibition curves and the respective fitting parameters of diclofenac, ibuprofen, metoprolol and propranolol. These four potent growth inhibitor drugs were selected for the binary mixture studies (see Section 3.2 and Appendix A).

concentrations (0.5 TU and 0.75 TU mixture) additivity was observed while at the higher mixture concentrations (1.5 TU, 1.75 TU and 2 TU) antagonism was found (Appendix A, Table A4). At the intermediate concentrations (1 TU and 1.25 TU) antagonism was predominant, but additivity was also observed. The *ibuprofen + propranolol* mixtures showed similar interaction profile to the ibuprofen + metoprolol combination except that additivity was more frequent at the intermediate mixture concentrations (1 TU and 1.25 TU) and antagonism occurred also at low mixture concentrations (0.5 TU and 0.75 TU) (Appendix A, Table A5). Finally, with the *metoprolol + ibuprofen* mixtures antagonisms were observed in almost all cases similarly to the described one for the diclofenac + ibuprofen combination except for three mixtures (0.25 TU metoprolol + 0.25 TU propranolol; 0.5 TU metoprolol + 0.25 TU propranolol; 0.5 TU metoprolol + 0.5 TU propranolol) where additivity was observed (Appendix A, Table A6).

3.3. Chemotactic effects of single pharmaceuticals at sublethal concentrations

Migratory responses of *T. pyriformis* elicited by the fourteen pharmaceuticals were tested in sublethal concentrations in the 10^{-15}–10^{-6} M range that covered environmentally relevant concentrations (below 10^{-9} M). It was found that with the exception of naproxen all anti-inflammatory–analgesic pharmaceuticals exhibited significant chemorepellent character (Fig. 2). Acetyl salicylic acid had weak repellent action at 10^{-15} M (Chtx. Ind. = 84.2 ± 3.4%), 10^{-9} M (Chtx. Ind. = 80.4 ± 8.5%) and at 10^{-6} M (83.9 ± 7.6%). Diclofenac acted as a weak chemorepellent in a wide concentration range from 10^{-15} M to 10^{-9} M. This effect was significant at 10^{-15} M, 10^{-14} M and 10^{-11}–10^{-9} M; the respective Chtx. Ind. ranged from 73.8 ± 3.6% to 87.0 ± 2.9%. Fenoprofen also showed repellent character at 10^{-14} M (Chtx. Ind = 70.5 ± 9.2%) and ibuprofen was repellent at 10^{-7} M (Chtx. Ind = 66.3 ± 6.1%). Similarly, the analgesic paracetamol was repellent at 10^{-14}–10^{-11} M (Chtx. Ind = 77.2 ± 4.4%–84.8 ± 2.8%) yet at 10^{-13} M this effect was not significant. Naproxen in turn was the only anti-inflammatory drug that showed a weak, however, significantly chemoattractant effect at 10^{-13} M (Chtx. Ind = 127.3 ± 10.1%).

Three of the four antibiotics exhibited significant chemoattractant character (Fig. 3): erythromycin at 10^{-8} M (145.9 ± 18.3%); lincomycin at 10^{-15} and 10^{-9} M (Chtx. Ind = 170.3 ± 20.8% and 133.3 ± 9.2%); sulfamethoxazole at 10^{-11} M, 10^{-9} M and at 10^{-6} M (Chtx. Ind = 125.6 ± 9.2%–146.1 ± 20.3%). However, erythromycin and lincomycin had also repellent actions: erythromycin was repellent at 10^{-15} (Chtx. Ind = 72.1 ± 10.2%) whereas lincomycin was repellent at 10^{-6} M (Chtx. Ind = 78.3 ± 9.9%). Trimethoprim was neutral in the entire concentration range.

Among β-blockers (Fig. 4) metoprolol was repellent at 10^{-15} M, 10^{-14} M, 10^{-7} M and 10^{-6} M (Chtx. Ind = 67.0 ± 5.5%–82.3 ± 7.5%). Propranolol showed also repellent effects at 10^{-15} M. 10^{-11} M and 10^{-10} M (Chtx. Ind = 62.8 ± 2.0%–71.3 ± 4.2%) whereas timolol acted in similar way at 10^{-15} and 10^{-14} M (Chtx. Ind = 67.0 ± 10.5% and 75.1 ± 11.5% respectively) and had attractant character at 10^{-6} M (Chtx. Ind = 150.0 ± 13.3%). The iodinated X-ray contrast media Na-diatrizoate had also ambiguous, concentration dependent character since it was attractant at 10^{-14} M, 10^{-7} M and 10^{-6} M (Chtx. Ind = 137.5 ± 14.1%–209.3 ± 20.1%) and it was repellent at 10^{-13} M and 10^{-8} M (Chtx. Ind = 83.7 ± 4.4% and 64.3 ± 15.5% respectively).

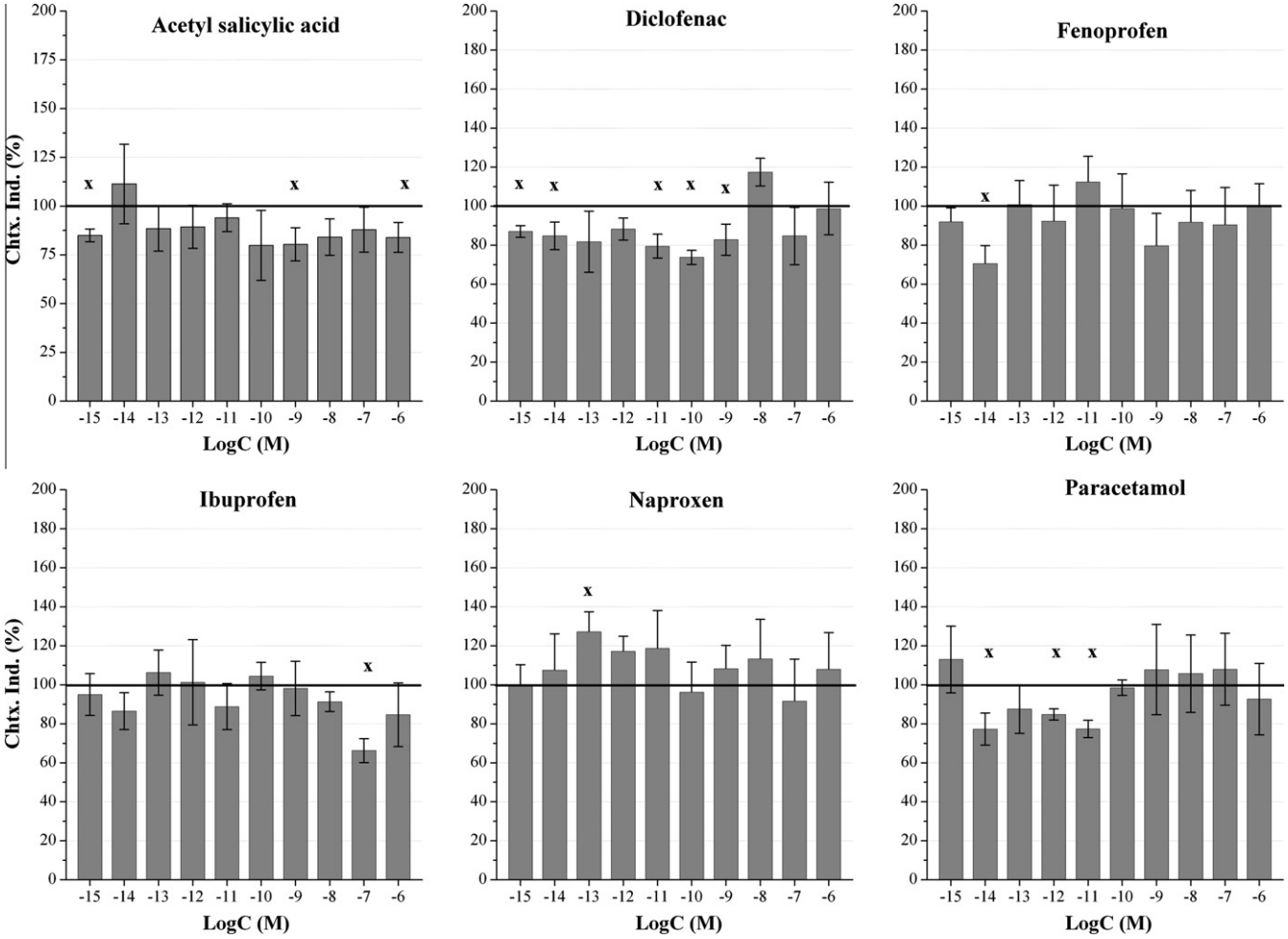

Fig. 2. Chemotactic responses of Tetrahymena cells induced by the five anti-inflammatory drugs acetyl salicylic acid, diclofenac, fenoprofen, ibuprofen, naproxen and the analgesic paracetamol. Significance level is x: $p < 0.05$.

4. Discussion

4.1. Growth inhibiting effects of single pharmaceuticals

Screening of the growth inhibiting effect of the 14 pharmaceuticals showed that *Tetrahymena pyriformis* was the most sensitive to treatment with β-blockers and NSAIDs. However, our results show that the observed effective concentrations are at least three orders of magnitude higher than environmental concentrations suggesting that acute toxic effects of these pharmaceuticals are improbable in the aquatic environment. Obtained EC_{50} values for the four selected drugs: 26.56 mg L^{-1} for diclofenac, 46.78 mg L^{-1} for ibuprofen, 608.85 mg L^{-1} for metoprolol and 4.82 mg L^{-1} for propranolol are comparable to literature data such as the study of Cleuvers (2003) which reported that the sensitivity of *Daphina* immobilization test, *Lemna* and *Desmodesmus* algal growth inhibition tests were in the same range (EC_{50} values varied between 5 mg L^{-1} and >320 mg L^{-1}). The reported order of toxicity diclofenac > ibuprofen > naproxen and propranolol > metoprolol was also identical to what was found by us. Comparison of this order with the most frequently evoked molecular descriptor related to the biodistribution and toxic effect of organic chemicals, the logarithmized octanol–water partitioning coefficient (Table 1), shows that in our experiment toxicity increased together with the $\log K_{ow}$: diclofenac ($\log K_{ow} = 4.4$) > ibuprofen ($\log K_{ow} = 3.5$) > naproxen ($\log K_{ow} = 3.3$) and propranolol ($\log K_{ow} = 3.0$) > metoprolol ($\log K_{ow} = 1.9$). This observation was consistent with the finding of (Cleuvers, 2004) who reported in a QSAR approach based study that NSAIDs acted on Daphnia and algae by nonpolar narcosis and that the higher the $\log K_{ow}$ of the substance the higher was its toxicity. However, in both the work of Cleuvers and the present study results were obtained using salt forms of the pharmaceuticals. Furthermore in an interspecies correlation experiment (Zhang et al., 2010) a poor interspecies correlation were found for hydrophobic substances between Tetrahymena and Daphnia or algae suggesting that depending on the organic chemical modes of uptake and action might differ from species to species (Zhang et al., 2010). Moreover, the establishment of quantitative relationship between certain molecular descriptor physicochemical parameters and biological effect (toxicity) may not be possible for all kinds of chemicals. In their work (Rudzok et al., 2011) for example found weak correlation between the toxicity and the $\log K_{ow}$ ($R^2 = 0.59$) value of organic contaminants such as pesticides, insecticides, and combustion products. Hence the application of QSAR approach might depend on the types of chemicals. Nevertheless, the other anti-inflammatory–analgesic pharmaceuticals seemed to "obey" to the above mentioned rule i.e. higher $\log K_{ow}$ means higher toxicity. Fenoprofen having a relatively high $\log K_{ow}$ value of 3.3 showed elevated toxicity whereas paracetamol possessing a low $\log K_{ow}$ value of 0.5 had no proliferation inhibiting effect at all. Similarly, the third β-blocker timolol with a $\log K_{ow} = 1.8$ which is very close to that of metoprolol ($\log K_{ow} = 1.9$), was almost as potent

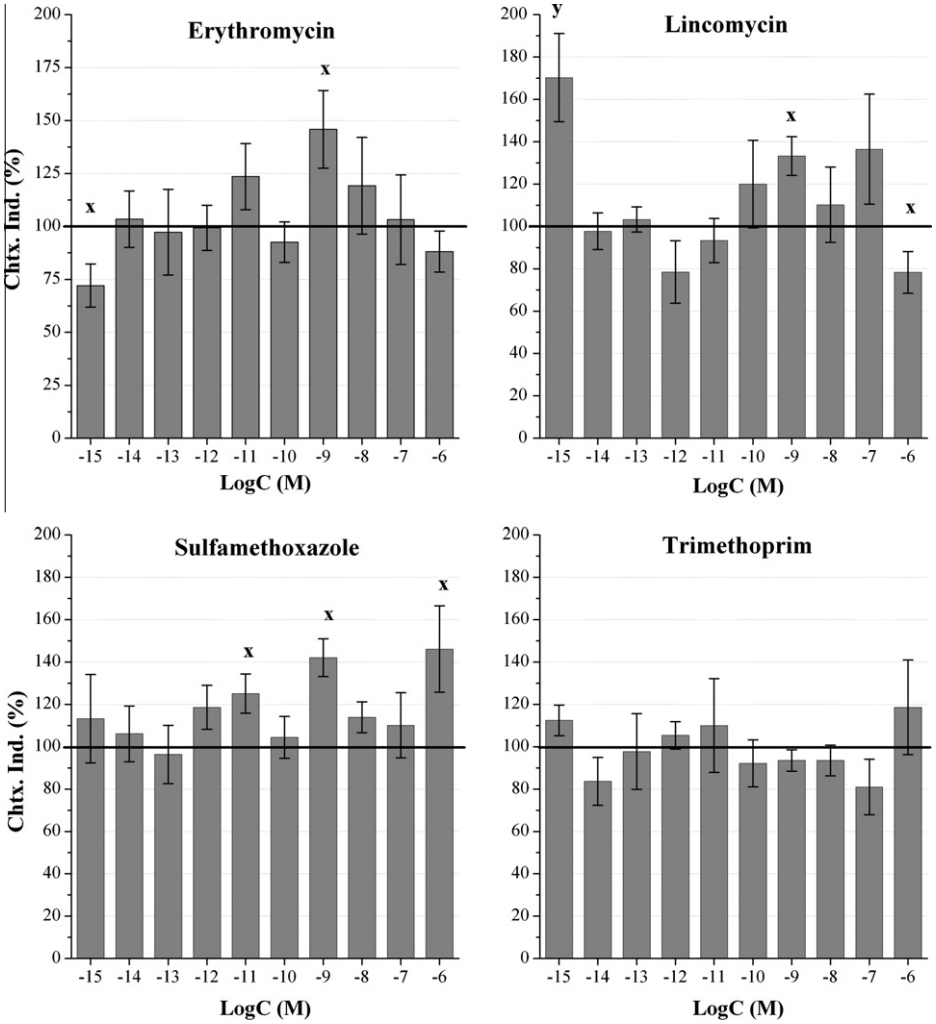

Fig. 3. Chemotactic responses of Tetrahymena cells elicited by the antibiotics: erythromycin, lincomycin, sulfamethoxazole and trimethoprim. Significance levels are x: $p < 0.05$ and y: $p < 0.01$.

proliferation inhibitor as the metoprolol. An interesting behavior, weak proliferation inhibition (~20%) at low concentrations (10^{-11}–10^{-8} M) but not at high concentrations was observed with acetyl salicylic acid ($\log K_{ow} = 1.2$) which was described by previous studies to inhibit Tetrahymena growth in high (10^{-4} M) concentration (Hokama et al., 1982). Different results may be attributed to the diverse experimental setup (such as cell density, culture volume and media).

In Tetrahymena, however, specific mode of action of the NSAIDs and β-blockers might not be excluded either since it posses the molecular target of both classes i.e. the β-adrenergic receptor (Blum, 1967) and cyclooxygenase (Hokama et al., 1982). However, genomic data are not yet available in the Tetrahymena Genome Database (http://www.ciliate.org) to compare them with the human homologues in order to draw some conclusions about their structure and affinity towards the human pharmaceuticals. For propranolol it is also known that its higher toxicity compared to other β-blockers is due to its strong membrane stabilizer potency that the other β-blockers lack (Fent et al., 2006).

The low sensitivity of Tetrahymena to antibiotics might be attributed to the fact that they are designed to act specifically on bacterial targets sites (Table 1). This might be the reason why erythromycin with the highest octanol–water partitioning coefficient ($\log K_{ow} = 2.7$) together with lincomycin ($\log K_{ow} = 0.2$) did not show proliferation inhibiting potency at all. In fact both substances inhibit bacterial protein synthesis via interfering specifically with the prokaryotic ribosome (Fürst, 2004). The two other antibiotics sulfamethoxazole ($\log K_{ow} = 0.9$) and trimethoprim ($\log K_{ow} = 0.9$) in turn exhibited weak proliferation inhibition effect in higher concentrations (10^{-7}–10^{-3} M). Originally, both of them are bacteriostatic due to their ability to abolish bacterial folate synthesis via the inhibition of the dihydropteroate syntethase and the dihydrofolate reductase respectively. However, even though with a much lower (about 10^4 fold weaker) affinity they are also able to interact with homologous eukaryotic targets (Fürst, 2004) such as the bifunctional dihydrofolate reductase-thymidylate synthase of Tetrahymena which has already been described not only from a functional point of view (Garrett et al., 1984) but also at the genomic level (the Tetrahymena Genome Database gene identifier is TTHERM_00312120). Protein BLAST alignment of the sequence reveals that it both the dihydrofolate reductase and the thymidylate synthase part contain several putative conserved domains. Homologous proteins were found in species at different levels of phylogeny such as plants (e.g. *Hordeum vulgarea*, the common barely) as well as in higher ranked vertebrates such as mice or human.

The X-ray contrast agent Na-diatrizoate ($\log K_{ow} = 1.8$) did not elicit proliferation inhibition either. Though little is known about the possible toxic effects of iodinated contrast media, a study using multiple biomarkers including phospholipids and adenosine triphosphate (ATP) reported that neither Na-diatrizoate nor its metabolites affected bacteria present in the sewage sludge (Haiß and Kümmerer, 2006).

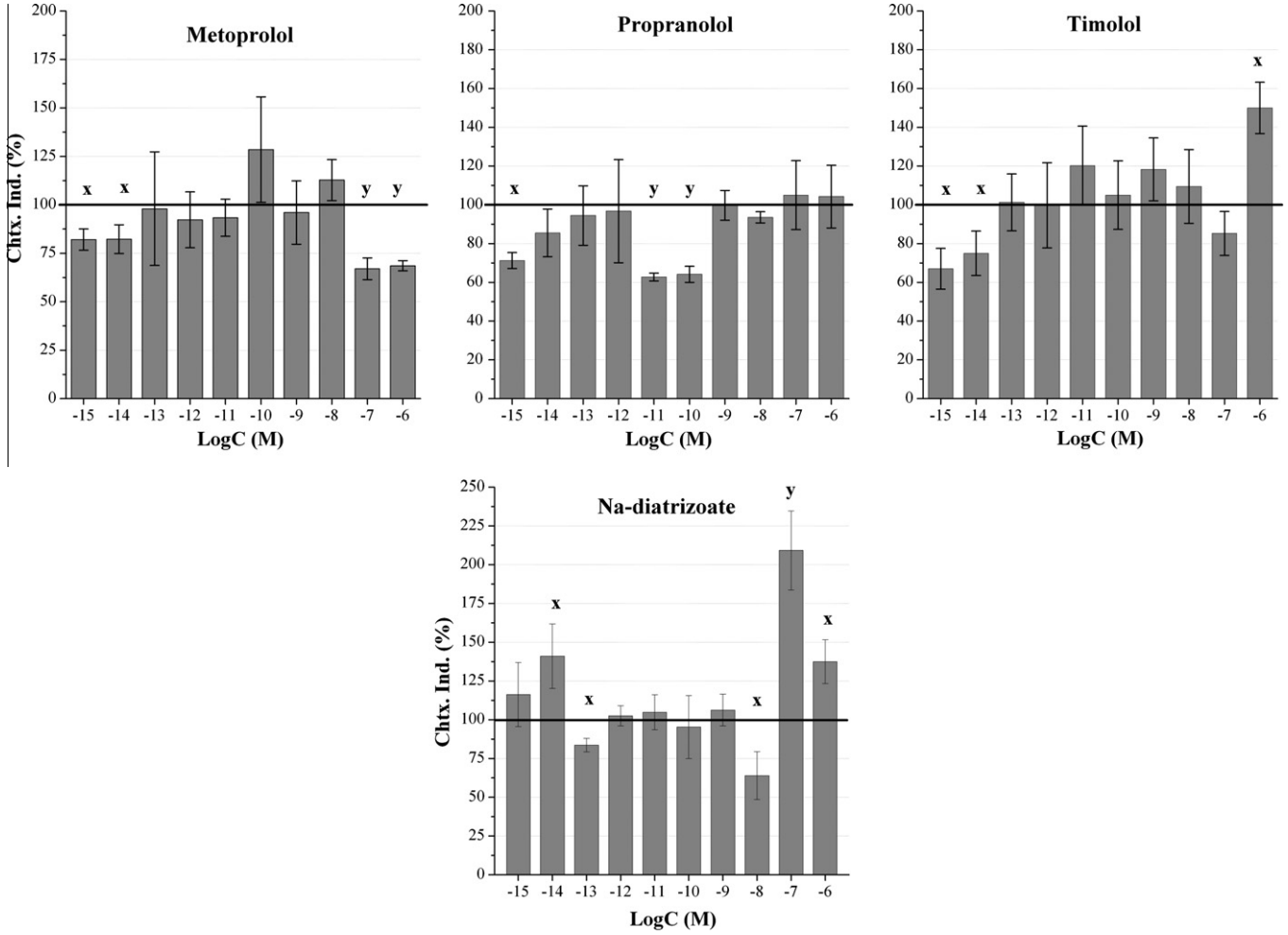

Fig. 4. Chemotactic responses of Tetrahymena cells induced by the β-blockers metoprolol, propranolol, timolol and the iodinated X-ray contrast media Na-diatrizoate. Significance levels are x: $p < 0.05$ and y: $p < 0.01$.

4.2. Combined action of diclofenac, ibuprofen, metoprolol and propranolol in binary mixtures

Predominant interaction type observed in mixtures was antagonism (in 59% of the combinations) and the frequency of its detection increased in general with the mixture concentration. It was found in all combinations with mixture concentration above 1.25 TU (except for the combination 1 TU diclofenac + 0.5 TU propranolol and the diclofenac + metoprolol mixtures). The predominance of antagonism in the higher concentration range might be explained by a potential competitive inhibition between the two pharmaceuticals acting on the same molecular target. However, it is more difficult to give an explanation to antagonisms observed between pharmaceuticals having different molecular targets. Anyhow, our results suggest that the concept of concentration addition validated for the NSAIDs in algal and Daphnia biotests (Cleuvers, 2004) cannot be fully adopted for Tetrahymena population inhibition assay. This may be due to the interspecies differences in the uptake and mechanism of action of these pharmaceuticals in these model organisms that was also stressed by Zhang et al. (2010). As a matter of fact, concentration additivity was observed only in the 37% of the mixtures. Synergism was the rarest type of interaction that was obtained only in 4% of the combinations and only in diclofenac + metoprolol mixtures (at relatively high mixture concentrations of 1.25 and 1.5 TU).

In order to explain the observed interaction type pattern we compared the concentration ratio of the two mixtures constituents and the observed interaction type as it was suggested for other contaminants such as heavy metals (Gallego et al., 2007) but no correlation was found. The complicated concentration dependent interaction type pattern seems to better corroborate with the findings Pomati et al. (2008) who studied the interactions of 13 environmentally relevant pharmaceuticals (such as ibuprofen, carbamazepine, and sulfamethoxazole) and found complex concentration dependent interaction profile difficult to predict or model.

4.3. Chemotactic responses of Tetrahymena to environmental concentrations of pharmaceuticals

Chemotaxis is a highly sensitive cell physiological response that can be elicited by a large variety of biologically active compounds at low concentrations. In environmental toxicology it was mainly studied in the context of bioremediation of polluted soil or ground waters. It was found that the chemotaxis of soil inhabiting microbial community members can enhance biodegradation of pollutants (e.g. aromatic hydrocarbons) by overcoming limitations in the bioavailability of these later (Parales and Harwood, 2002). Our results show that 13 of the tested pharmaceuticals altered significantly chemotactic responses of Tetrahymena in sublethal concentrations with trimethoprim being the only exception that were neutral. The respective effective concentrations were in the range of the environmental concentrations ($<10^{-9}$ M) except for the chemorepellent effect of ibuprofen. The chemotactic profile of the pharmaceuticals was not strictly correlated to their toxicity. The

most toxic drugs (diclofenac, metoprolol, propranolol) tended to have chemorepellent effect, but important differences could be observed in terms of effective concentration and the wideness of the chemorepellent concentration range. Moreover some pharmaceuticals such as timolol that proved to be quite toxic had not only chemorepellent but also attractant effect. Paracetamol, erythromycin and lincomycin in turn, that did not inhibit proliferation exhibited repellent effect.

Literature data about the chemotactic effect of these pharmaceuticals were mostly obtained in chemotaxis inhibition experiments where interaction of these drugs with the function of the immune system was studied using chemoattractant stimulated human polymorphonuclear leukocytes (PMNs). Diclofenac and ibuprofen were described to reduce chemotaxis of PMN and monocyte cells induced by potent chemoattractants like the substance P, transforming growth factor-β (Locatelli et al., 1993) and the bacterial tripeptide fMLF (Perianin et al., 1990). Similarly, the β-blockers metoprolol and propranolol but not timolol decreased the fMLF induced chemotaxis of neutrophils at 10^{-8} M (Djanani et al., 2003). The iodinated X-ray contrast agent was also reported to inhibit PMN chemotaxis induced by fMLF at micromolar concentration possibly by interacting with the specific fMLF receptor (Levesque et al., 1992) as well as erythromycin, that reduced *in vivo* and *in vitro* neutrophil migration in inflammatory conditions (Oda et al., 1994). Interestingly, the strong chemoattractant potential of fMLF (Chtx. Ind. >250% at 10^{-8} M) towards Tetrahymena was also described (Kőhidai et al., 2003). This would allow us to modify experimental setup in the future and to test the inhibiting effects of these pharmaceuticals on fMLF induced chemotaxis that might result in a concentration – migratory response relationship that is more suitable for quantitative analysis than the results presented here. Even if the chemotactic response of Tetrahymena is a highly sensitive assay endpoint, most often the obtained concentration–response relationship is not monotonic as it was described for a large number of compounds, e.g. cytokines (Köhidai and Csaba, 1998) or the vasoconstrictor peptide endothelin-1 (Kőhidai and Csaba, 1995). This observation can be explained by the existence of more than one type of receptors with different affinities for the same ligand (e.g. endothelin receptor 1 and 2) (Takeshi et al., 1992). The saturation level of chemotaxis receptors can also determine chemotactic responsiveness of the cell: low and over saturated states can result weak responses and consequently non-monotonic concentration dependence of chemotaxis (Wilkinson and Lackie, 1983). Alternating migration inhibiting and inducing behavior is general and it was observed amongst other in human neutrophil granulocytes in response to pharmaceuticals (e.g. propranolol) and pesticide mixtures (Calabrese, 2005a). These results were explained by hormesis that is a general phenomenon at all the levels of phylogeny and was observed with various endpoints. Although the underlying mechanisms still need elucidation, the explanation is linked again to the simultaneous presence of low and high affinity receptor populations responding to a ligand. The ecotoxicological examples, relevance and consequences of this phenomenon were reviewed by Calabrese (2005b).

All in all, in its present form our chemotaxis assay should be used as a qualitative assay that nevertheless can indicate if a pharmaceutical have sublethal, behavior modifying effect at environmental concentrations.

5. Conclusions

Our data gained on *Tetrahymena pyriformis* GL underline the significance of this eukaryotic ciliate model as a proper bioindicator of environmental pollution. The growth inhibition and binary toxicity assays demonstrated that essential physiological functions of this model can be finely and selectively modulated by pollutants and their mixtures. Furthermore the chemotactic responsiveness should be also considered as a significant evaluation criteria in pollution assays. However, the use of some specific biochemical endpoints could also help to better understand the mechanism of the action of aquatic contaminant pharmaceuticals in Tetrahymena as well as in protozoan level in general.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2012.05.058.

References

Berenbaum, M.C., 1985. The expected effect of a combination of agents: the general solution. J. Theor. Biol. 114, 413–431.

Blum, J.J., 1967. An adrenergic control system in Tetrahymena. Proc. Natl. Acad. Sci. USA 58, 81–88.

Bogaerts, P., Bohatier, J., Bonnemoy, F., 2001. Use of the ciliated protozoan *Tetrahymena pyriformis* for the assessment of toxicity and quantitative structure–activity relationships of xenobiotics: comparison with the MicroTox test. Ecotoxicol. Environ. Saf. 49, 293–301.

Calabrese, E.J., 2005a. Hormetic dose-response relationships in immunology: occurrence, quantitative features of the dose response, mechanistic foundations, and clinical implications. Crit. Rev. Toxicol. 35, 89–295.

Calabrese, E.J., 2005b. Paradigm lost, paradigm found: the re-emergence of hormesis as a fundamental dose response model in the toxicological sciences. Environ. Pollut. 138, 378–411.

Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142, 185–194.

Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicol. Environ. Saf. 59, 309–315.

Csaba, G., Németh, G., 1980. Effect of hormones and their precursors on protozoa—the selective responsiveness of tetrahymena. Comp. Biochem. Physio.–C: Comp. Pharmacol. 65, 387–390.

Dayeh, V.R., Lynn, D.H., Bols, N.C., 2005. Cytotoxicity of metals common in mining effluent to rainbow trout cell lines and to the ciliated protozoan, *Tetrahymena thermophila*. Toxicol. in Vitro, 399–410.

Djanani, A., Kaneider, N.C., Meierhofer, C., Sturn, D., Dunzendorfer, S., Allmeier, H., Wiedermann, C.J., 2003. Inhibition of neutrophil migration and oxygen free radical release by metipranolol and timolol. Pharmacology 68, 198–203.

Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122–159.

Fürst, Z., 2004. Farmakológia. Medicina Könyvkiadó Rt, Budapest.

Gabet-Giraud, V., Miège, C., Choubert, J.M., Ruel, S.M., Coquery, M., 2010. Occurrence and removal of estrogens and beta blockers by various processes in wastewater treatment plants. Sci. Total Environ. 408, 4257–4269.

Gallego, A., Martín-González, A., Ortega, R., Gutiérrez, J.C., 2007. Flow cytometry assessment of cytotoxicity and reactive oxygen species generation by single and binary mixtures of cadmium, zinc and copper on populations of the ciliated protozoan *Tetrahymena thermophila*. Chemosphere 68, 647–661.

Garrett, C.E., Coderre, J.A., Meek, T.D., Garvey, E.P., Claman, D.M., Beverley, S.M., Santi, D.V., 1984. A bifunctional thymidylate synthetase-dihydrofolate reductase in protozoa. Mol. Biochem. Parasitol. 11, 257–265.

Gerhardt, A., Ud-Daula, A., Schramm, K.-W., 2010. Tetrahymena spp. (Protista, Ciliophora) as test species in rapid multilevel ecotoxicity tests. Anglais 49, 271–280.

Haiß, A., Kümmerer, K., 2006. Biodegradability of the X-ray contrast compound diatrizoic acid, identification of aerobic degradation products and effects against sewage sludge micro-organisms. Chemosphere 62, 294–302.

Hokama, Y., Yokochi, L., Abad, M.A., Shigemura, L., Kimura, L.H., Okano, C., Chou, S.C., 1982. Presence of prostaglandins (PGs) in *Tetrahymena pyriformis*, GL and the effect of aspirin. Res. Commun. Chem. Pathol. Pharmacol. 38, 169–172.

Kőhidai, L., 1995. Method for determination of chemoattraction in *Tetrahymena pyriformis*. Curr. Microbiol. 30, 251–253.

Köhidai, L., Csaba, G., 1998. Chemotaxis and chemotactic selection induced with cytokines (IL-8, RANTES and TNF-alpha) in the unicellular Tetrahymena pyriformis. Cytokine 10, 481–486.

Kőhidai, L., Csaba, G., 1995. Effects of the mammalian vasoconstrictor peptide, endothelin-1, on *Tetrahymena pyriformis* GL, and the immunocytological detection of endogenous endothelin-like activity. Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 111, 311–316.

Kőhidai, L., Török, K., Illyés, E., Tamási, J., Sebestyén, F., Láng, O., Csaba, G., Hudecz, F., 2003. Characterization of chemotactic ability of peptides containing N-formyl-methionyl residues in Tetrahymena fMLP as a targeting ligand. Cell Biol. Int. 27, 695–700.

Kümmerer, K., 2009. Antibiotics in the aquatic environment – a review – Part I. Chemosphere 75, 417–434.

Levesque, L., Gaudreault, R.C., Marceau, F., 1992. Comparison of two classes of non-peptide drugs as antagonists of neutrophil receptors for f-Met-Leu-Phe: Pyrazolons and iodinated radiographic contrast agents. Biochem. Pharmacol. 43, 553–560.

Locatelli, L., Sacerdote, P., Mantegazza, P., Panerai, A.E., 1993. Effect of ibuprofen and diclofenac on the chemotaxis induced by substance P and transforming growth factor-β on human monocytes and polymorphonuclear cells. Int. J. Immunopharmacol. 15, 833–838.

Oda, H., Kadota, J.-i., Kohno, S., Hara, K., 1994. Erythromycin inhibits neutrophil chemotaxis in bronchoalveoli of diffuse panbronchiolitis. Chest 106, 1116–1123.

Pal, A., Gin, K.Y.-H., Lin, A.Y.-C., Reinhard, M., 2010. Impacts of emerging organic contaminants on freshwater resources: review of recent occurrences, sources, fate and effects. Sci. Total Environ. 408, 6062–6069.

Parales, R.E., Harwood, C.S., 2002. Bacterial chemotaxis to pollutants and plant-derived aromatic molecules. Curr. Opin. Microbiol., 266–273.

Perianin, A., Giroud, J.P., Hakim, J., 1990. Stimulation of human polymorphonuclear leukocytes potentiates the uptake of diclofenac and the inhibition of chemotaxis. Biochem. Pharmacol. 40, 2039–2045.

Pomati, F., Orlandi, C., Clerici, M., Luciani, F., Zuccato, E., 2008. Effects and interactions in an environmentally relevant mixture of pharmaceuticals. Toxicol. Sci. 102, 129–137.

Putschew, A., Wischnack, S., Jekel, M., 2000. Occurrence of triiodinated X-ray contrast agents in the aquatic environment. Sci. Total Environ. 255, 129–134.

Rudzok, S., Krejči, S., Graebsch, C., Herbarth, O., Mueller, A., Bauer, M., 2011. Toxicity profiles of four metals and 17 xenobiotics in the human hepatoma cell line HepG2 and the protozoa *Tetrahymena pyriformis*—a comparison. Environ. Toxicol. 26, 171–186.

Sauvant, N.P., Pepin, D., Piccinni, E., 1999. Tetrahymena pyriformis: a tool for toxicological studies. Chemosphere: A Rev. 38, 1631–1669.

Schiess, N., Csaba, G., Kőhidai, L., 2001. Chemotactic selection with insulin, di-iodotyrosine and histamine alters the phagocytotic responsiveness of Tetrahymena. Comp. Biochem. Phys. C 128, 521–530.

Sprague, J.B., 1970. Measurement of pollutant toxicity to fish. II. Utilizing and applying bioassay results. Water Res. 4, 3–32.

Takeshi, S., Masashi, Y., Tomoh, M., 1992. Molecular characterization of endothelin receptors. Trends. Pharmacol. Sci. 13, 103–108.

Ternes, T.A., 2001. Analytical methods for the determination of pharmaceuticals in aqueous environmental samples. Trends Anal. Chem. 20, 419–434.

Wilkinson, P.C., Lackie, J.M., 1983. The influence of contact guidance on chemotaxis of human neutrophil leukocytes. Exp. Cell Res. 145, 255–264.

Zhang, X.J., Qin, H.W., Su, L.M., Qin, W.C., Zou, M.Y., Sheng, L.X., Zhao, Y.H., Abraham, M.H., 2010. Interspecies correlations of toxicity to eight aquatic organisms: theoretical considerations. Sci. Total Environ. 408, 4549–4555.

Zuccato, E., Castiglioni, S., Fanelli, R., 2005. Identification of the pharmaceuticals for human use contaminating the Italian aquatic environment. J. Hazard. Mater. 122, 205–209.