

## Input and behaviour of alkylphenolic endocrine-disrupting contaminants in a stratified estuary and coastal waters

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### INTRODUCTION

Aromatic surfactants of alkylphenol polyethoxylate (APnEO) type with estimated annual worldwide production rate of about  $6 \times 10^5$  tons belong to the most popular surfactant classes. Due to their widespread use in such high volumes and considerable toxicities to aquatic life, APnEO should be considered potentially important environmental contaminants (Servos, 1999; Ying *et al.*, 2002).

It was shown in model laboratory experiments as well as in sewage treatment plants and various types of ambient waters that APnEO exhibit a complex metabolic behaviour, yielding a variety of relatively stable metabolic products. Biotransformation of APnEO is very complex and usually starts at the hydrophylic part of the molecule, resulting in a rather unique metabolic pathway, which is characterised by the formation of various stable intermediates (Ahel *et al.*, 1994) (Figure 1). Some of these metabolites are more lipophylic and therefore more toxic to aquatic life than the parent molecules (see review by Servos, 1999). In the last decade, the interest in ecotoxicological effects of these lipophylic metabolites has dramatically increased because of their proven endocrine-disrupting potential (Jobling and Sumpter, 1993; Jobling *et al.*, 1996; Metcalfe *et al.*, 2001; Johnson and Sumpter, 2003). The most recent studies have shown that APnEO intermediates with shorter EO-chains are further transformed by microbial carboxylation of highly branched alkyl-chain, yielding a suite of more polar metabolites, which also exhibit a significant persistence (Ding *et al.*, 1996; Di Corcia *et al.*, 1998). However, these metabolic products are less toxic than the metabolites containing alkylphenol moiety, while their potential for endocrine disruption seems to be insignificant (Johnson and Sumpter, 2003).

Owing to their hydrophobic moiety, APnEO show a significant affinity for suspended particles, which results in a widespread presence of surfactant residues in aquatic sediments and biota (Bennie, 1999). The adsorption onto sediment and bioaccumulation are particularly pronounced for the lipophylic degradation products of APnEO such as nonylphenol (NP), nonylphenol monoethoxylate (NP1EO), and nonylphenol diethoxylate (NP2EO).

Coastal and estuarine waters receive large quantities of aromatic surfactants, either directly from municipal wastewaters or indirectly from polluted rivers. In the past decade, there have been an increasing number of papers dealing with the behaviour and fate of APnEO in coastal and estuarine environments (Marcomini *et al.*, 1990; Blackburn *et al.*, 1999; Isobe *et al.*, 2001; Ferguson *et al.*, 2003; Jonkers *et al.*, 2003). In estuaries, most of the published data deal with well-mixed macrotidal estuaries, while the studies in stratified estuaries, characteristic of the Mediterranean Sea, are relatively scarce. There are two major motivations to study behaviour and

fate of surfactants in estuarine environments. Many of the world estuaries are densely populated and represent areas of intensive and often mutually conflicting anthropogenic activities including fishery, industry, navigation and tourism. On the other hand, estuaries are well known as important regions of biological diversity and as such, potentially highly vulnerable to environmental stress imposed by different human activities, including those characterised by extensive introduction of man-made chemicals.

This paper summarizes our studies (Kvestak and Ahel, 1994; Kvestak *et al.*, 1994; Kvestak and Ahel, 1995) conducted in the estuary of the Krka River, Croatia, and discusses environmental behaviour and fate of APnEO and their metabolites in this unique system characterised by sharp salinity gradients. In addition, the results of the recent screening of endocrine-disrupting compounds, including surfactant-derived alkylphenolic compounds and bisphenol A, in Croatian coastal waters are also presented.

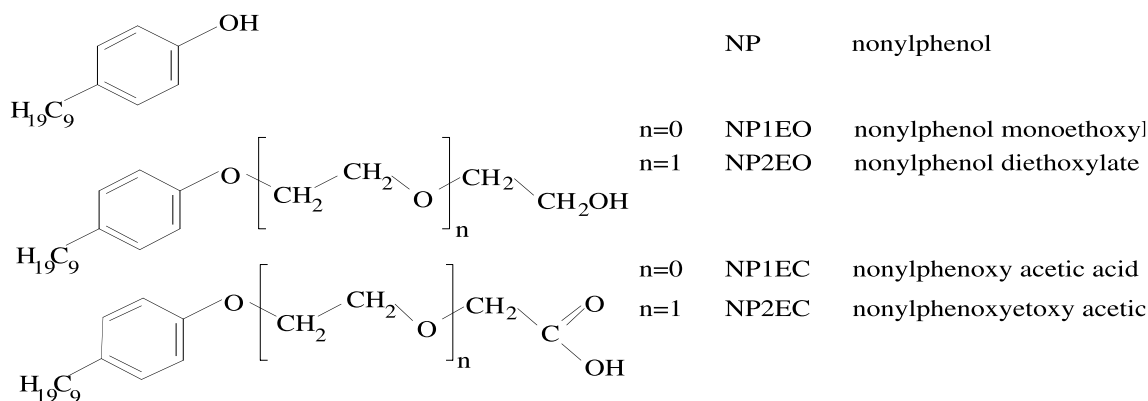


Fig. 1. Structures and acronyms of the most important estrogenic metabolites of nonylphenol polyethoxylates.

### SITUATION AND HYDROGRAPHIC FEATURES OF THE PERMANENTLY STRATIFIED KRKA RIVER ESTUARY

The studies presented in this paper were carried out in the Krka River estuary situated in the middle part of the eastern Adriatic coast. The total length of the estuary is about 30 km and the depth varies from few meters, immediately below the waterfalls, to 40 meters at the sea-end of the estuary (Sibenik Harbour). Owing to its unique hydrographic features, the Krka River estuary belongs to the most intensively investigated estuaries of the Mediterranean Sea with a special emphasis on the processes that occur at estuarine boundaries. The Krka River estuary is a typical karstic estuary characterised by low terrigenous input of suspended materials and conspicuous vertical salinity gradients, resulting in a very stable stratification throughout the year (Figure 2). The upper freshwater and lower seawater layers are separated with a visible interface situated at the halocline, which contains an organic film formed mainly by accumulation of plankton-derived organic matter (Zutic and Legovic, 1987). The depth of the halocline fluctuates seasonally, depending on the river flow, between 0.2 and 6 m.

It was shown that such stratified estuaries are particularly suitable for studies of environmental behaviour of different constituents since the entire range of estuarine master variables can be achieved at one single station, which significantly simplifies the required sampling strategy. On the other hand, the Krka River estuary shows a conspicuous change of anthropogenic pressure along its longitudinal profile. The largest part of the estuary is a pristine environment situated within the borders of a National Park area with only few smaller settlements and very limited industrial activities, while the main source of anthropogenic input are wastewaters of the city of Sibenik (40000 inhabitants), which enter the estuary in its lower part, i.e. in the area of Sibenik Harbour. Municipal and industrial wastewaters are discharged into the estuary without any pretreatment through several outlets along the northern shoreline of the Sibenik Harbour basin.

Since 1989, several sampling campaigns have been performed, comprising sampling of wastewaters and estuarine water column. In order to determine spreading of wastewater plume

into the Sibenik Harbour, water samples were collected at different distances from the major sewage outlets at depths characteristic of brackish (0.5 m) and saline (6 m) layers. A more detailed sampling on the vertical profile of the water column, with a special emphasis on the brackish water-seawater interface, was performed at the station E4A, which is situated in the middle part of the Sibenik Harbour.

A nation-wide screening of alkylphenolic compounds was performed on several hot-spots along the eastern Adriatic coast as a pilot-study within the national monitoring programme (Project Jadran).

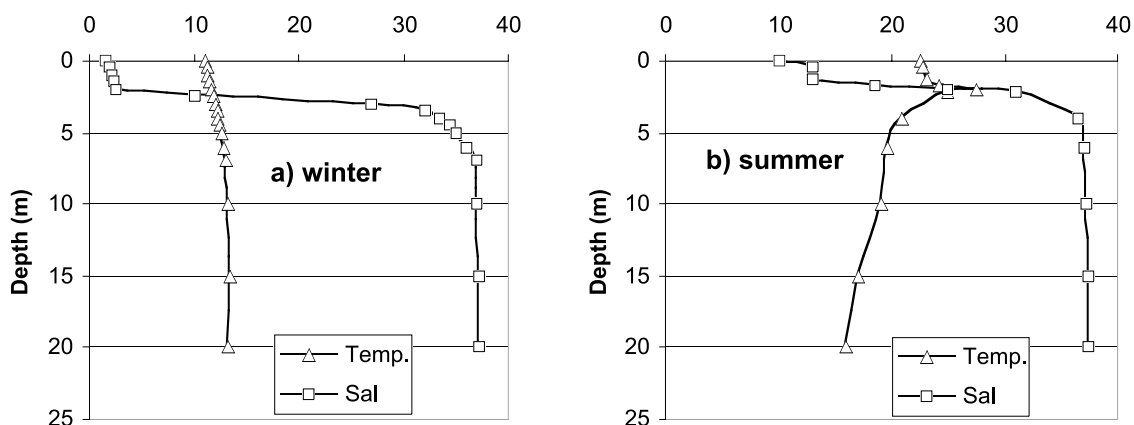


Fig. 2. Typical vertical profiles of temperature ( $^{\circ}\text{C}$ ) and salinity (PSU) in the water column of the Krka River estuary in winter and summer (Grzetic, 1990).

## METHODS

NPnEO, including their stable metabolic products, were determined using an approach based on HPLC separation followed by spectrofluorimetric detection, which involved both reversed phase (separation of homologues and isomers) and normal phase (separation of oligomers) systems (Ahel and Giger, 1985a; Ahel and Giger, 1985b; Ahel *et al.*, 2000). Liquid chromatography/tandem mass spectrometry (LC/MS/MS) was used for identification and confirmation purposes, as well for highly specific quantitative determination of alkylphenols and alkylphenol carboxylic acids (Petrovic *et al.*, 2003). In order to investigate the partitioning behaviour of surfactant residues, water samples were previously filtered through glass fiber filters (GF/F) and the dissolved and particulate fractions were further separately processed. Briefly, dissolved fraction of both classes of aromatic surfactants was enriched from wastewater and estuarine waters using  $\text{C}_{18}$  solid-phase extraction, while the particulate fraction was extracted using an ultrasonically enhanced extraction. Lipophylic metabolites of NPnEO (NP, NP1EO and NP2EO) were determined by an alternative enrichment procedure, using a continuous steam distillation/solvent extraction in a special apparatus, followed by a direct analysis of the cyclohexane extracts by normal phase HPLC (Ahel and Giger, 1985a).

In addition to the determination of aromatic surfactant residues in the estuary, the die-away biodegradation experiments were performed to assess the behaviour and fate of the target compounds (Ahel and Kvestak, 1995). The experiments were performed using autochthonous microbial populations, which were sampled in different estuarine compartments as well as in different seasons. The biodegradation media were always kept at temperatures identical to those found in the ambient water at the moment of sampling.

## CHARACTERISATION OF SURFACTANT INPUT

The input of APnEO surfactants into the Sibenik Harbour was determined by analysing wastewaters from all major outlets of the city of Sibenik (Kvestak *et al.*, 1994). The homologue- and oligomer-compositions (Figure 3) found in wastewater extracts revealed the presence of common commercial mixtures of APnEO, which have been partially changed due to the physico-chemical partitioning and biotransformation in the sewer system. It should be stressed that a

significant percentage of APnEO (6-60 %) entering Sibenik Harbour via municipal wastewaters is bound to sewage particles. This fact markedly influences their fate in the estuary. The determination of aromatic nonionic surfactants in wastewater extracts by reversed-phase HPLC (Ahel and Giger, 1985b) indicated a strong predominance of nonyl homologues, while the presence of octyl homologues was insignificant (<5 %). The oligomer composition of NPnEO in the total wastewater samples displays a great deal of similarity to those commercial mixtures that are commonly used in detergent formulations, which are characterised by an oligomer distribution maximum at NP10EO.

The comparison of distribution patterns in the dissolved and particulate phases shows a pronounced selectivity in the partitioning of individual oligomers. The oligomer composition in the particulate phase is characterised by an enhanced abundance of lower oligomers, resulting in oligomer distributions that show typical maxima between NP6-9EO. This situation can be explained by decreased water solubilities and, consequently, increased lipophylicities (Ahel and Giger, 1993) of the lower oligomers. The observed change in the oligomer distribution patterns depends on the concentration of suspended solids in the wastewater sample. The typical maximum of oligomer distribution in the dissolved phase displays a shift of the maximum towards higher oligomers (NP11EO-NP12EO). It should be noted that the oligomer distributions presented in Figure 3 show only the distribution of higher oligomers ( $nEO > 3$ ), which are considered parent compounds (Ahel *et al.*, 1994). In addition to these parent oligomers, their lipophylic metabolites, including NP, NP1EO and NP2EO, were detected in all samples in lower but significant concentrations (typically below 10  $\mu\text{g/L}$  of the individual metabolite). However, due to a rather short sewer system (residence time is estimated to be less than 1 hour), only a minor part of the parent compounds was biotransformed and the contribution of the lipophylic metabolites to the total concentration of nonylphenolic compounds did not exceed 5 %.

It is interesting to note that the concentration of aromatic surfactants varied significantly during the period covered by these investigations (1989-2003). In the late eighties, the average levels of the NPnEO were rather high (approximately 0.5 mg/L), suggesting an extensive usage of NPnEO in that period. Assuming an average discharge of municipal wastewaters of about 0.3  $\text{m}^3/\text{s}$ , the input of NPnEO was estimated at 5 tons per year. As a consequence of risk reduction measures, which were introduced in Western Europe, as well as reduced industrial activities during and after the war in Croatia (1991-1995), the concentration of NPnEO decreased below 0.1 mg/L. However, they still represent ubiquitous contaminants in Croatian wastewaters.

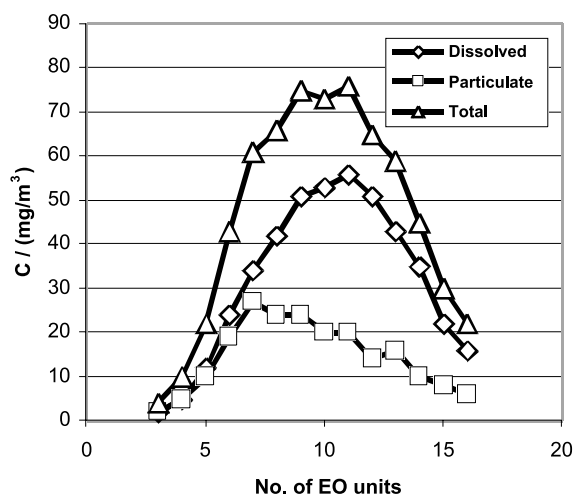


Fig. 3. Distribution of oligomers of nonylphenol polyethoxylates (NPnEO) in dissolved and particulate fractions of municipal wastewaters in Sibenik Harbour.

#### DISTRIBUTION IN THE WASTEWATER PLUME

The spreading of the wastewater plume in the Sibenik Harbour was investigated by taking the samples at different distances from the main outlet at two different depths characteristic of the brackish compartment (0.5 m; salinities <20 PSU) and marine compartment (6 m; salinities >37 PSU) of the estuary. The results indicate that spreading of wastewaters occurs almost exclusively

in the brackish layer, while the underlying saline layer remains hardly affected. Figure 4 shows the distribution of NPnEO in the wastewater plume along with the most important group of anionic surfactants, linear alkylbenzene sulphonates (LAS). As can be seen, there is a strong reduction (down to 1-10  $\mu\text{g/L}$ ) of the surfactant concentrations in the closest proximity (25 m) of the outlet. After a distance of only 100 m the concentration of both NPnEO and LAS in the plume dropped down below 2  $\mu\text{g/L}$ . The observed effect depends strongly on wind conditions, which can enhance the velocity and direction of surface currents as well as mixing of the brackish and saline layers (Legovic *et al.*, 1991). Such a fast reduction of surfactant concentration with increasing distance from the outlets can mainly be assigned to a very fast dilution; however, differences in the distribution patterns of individual homologues and/or oligomers suggest the importance of some other mechanisms that lead to the elimination of parent compounds. If the relative contribution of lipophylic metabolites in the total nonylphenolic compounds is plotted versus distance from the outlet (Figure 5), one can see that their percentage in the plume significantly increases (up to 54 %). This indicates possible importance of some oligomer-specific elimination process in the plume, most probably biotransformation of the parent NPnEO. Alternative mechanism, i.e. fast sedimentation of surfactant molecules with sewage-derived particles, is less likely since this process would tend to decrease the relative percentage of lipophylic metabolites. However, Marcomini *et al.* (1990) have shown that the association of the lipophylic metabolites with suspended particles is an important process leading to significant concentrations in the uppermost sediment layer.

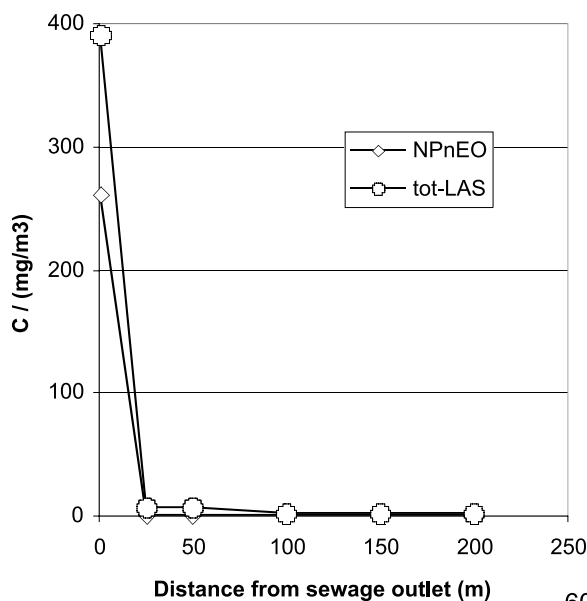
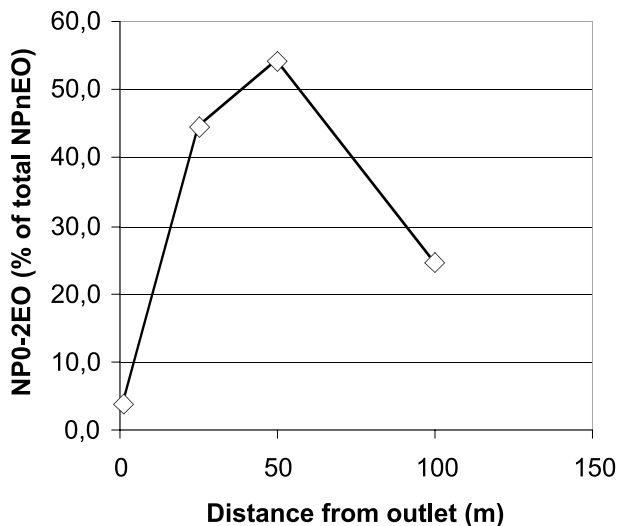


Fig. 4. Dilution of nonylphenol polyethoxylates (NPnEO) and linear alkylbenzene sulphonates (LAS) in the wastewater plume near the outlet in the Sibenik Harbour.

Fig. 5. Relationship between the parent nonylphenol polyethoxylates (NPnEO) and their lipophylic metabolites (NP0-2) in the wastewater plume in the Sibenik Harbour.



### DISTRIBUTION ON THE VERTICAL PROFILE

The most characteristic feature of the surfactant distribution in a microtidal estuary is the distribution pattern on the vertical profile of the water column (Figure 6). The maximal concentrations of both NPnEO and LAS are observed at estuarine phase boundaries, i.e. in the surface microlayer (air-brackish water interface) and at the brackish-seawater interface. Such a tendency of aromatic surfactants to accumulate at the phase boundaries is in agreement with their amphiphilic nature. The enrichment factors in the surface microlayer (sampled with the Garrett net) were in the range from 3.2-19.5, while the apparent enrichment at the brackish water-seawater interface was smaller. However, comparatively lower enrichment factors at the brackish water-seawater interface could be just a consequence of insufficient resolution of the procedure that was used for the sampling of organic films at the brackish water-sea water interface (2 cm), as compared to the well-known Garrett net technique. It should be noted that the concentration of both NPnEO and LAS in underlying seawater layer are very low, indicating that their transport through the brackish water-saline water interface is greatly reduced. The interface prevents both the mixing of the two layers as well as a vertical flux of suspended particles (Zutic and Legovic, 1987). It is interesting to note that a similar vertical concentration profile of NPnEO-derived compounds, characterised by a pronounced maximum at the boundary of two physically different water masses, was observed in Lake Geneva, with the maximum situated at the thermocline (Ahel, 1987).

The homologue and/or oligomer compositions of nonylphenolic compounds vary along the vertical profile, suggesting that significant changes occur during the passage through the interface. As indicated in Figure 6, the concentration of lipophylic metabolites of NPnEO shows a very sharp maximum at the halocline, reaching a high share of over 60 % of the total nonylphenolic compounds. These results indicate that the marine interfaces can be regarded as the critical sites of enhanced exposure to the lipophylic metabolites, which represent the most toxic and endocrine-disrupting species derived from NPnEO surfactants.

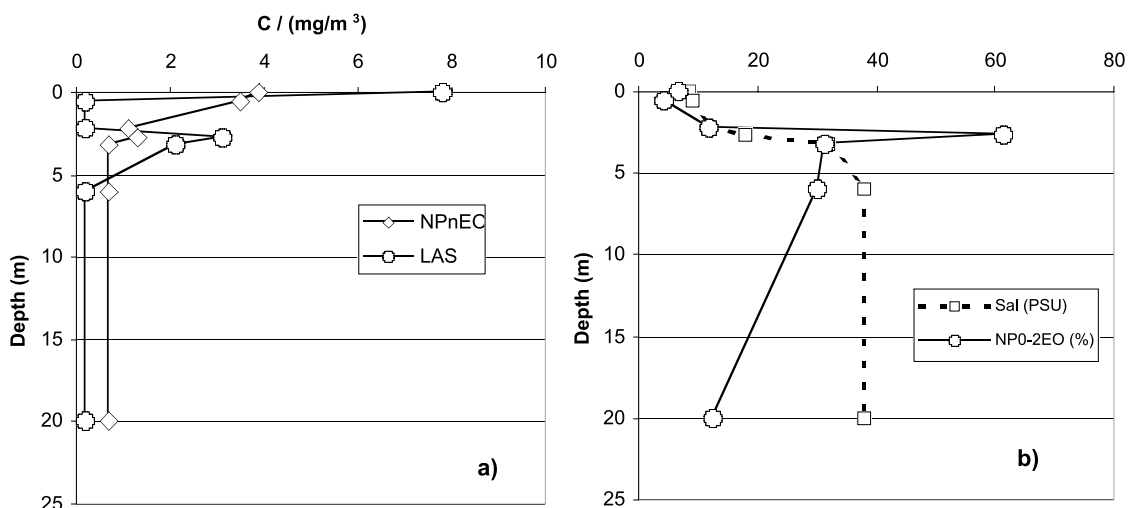


Fig. 6. Vertical profiles of surfactant residues in the water column of a stratified estuary: (a) distribution of nonylphenol polyethoxylates (NPnEO) and linear alkylbenzene sulphonates (LAS) and (b) percentage of lipophylic metabolites in the total NPnEO.

### BIOTRANSFORMATION OF AROMATIC SURFACTANTS

The biotransformation processes were shown to be seasonally dependent and the seasonal differences are more clearly reflected in the oligomer composition than in the homologue patterns. For NPnEO oligomers, one could clearly separate the effects of biotransformation and adsorption, since biotransformation is more effective for the higher oligomers, while the lower oligomers exhibit a stronger affinity for adsorption. The corresponding average number of EO



units in summer and winter are typically found in the ranges of 7 to 9 and 9 to 11, respectively, which indicates a more efficient degradation during summer. Field observations on seasonally dependent transformation were confirmed by controlled laboratory experiments, which were conducted using natural microbial populations, originating from both brackish and saline estuarine compartments. The results presented in Table 1 show the estimated biotransformation rates of NPnEO in comparison to those for LAS under different temperature conditions. Estimated overall biotransformation rate constants for NPnEO (0.01 to 0.28 days<sup>-1</sup>) are generally in a similar range like those for LAS; however, a more detailed analysis shows that NPnEO are more resistant to biodegradation by microbial populations from the saline layer and/or under lower temperatures. In addition, the model experiments have showed that biotransformation of NPnEO results in the formation of persistent metabolites, including NP1EO and NP2EO, as well as corresponding carboxylic acids (Kvestak and Ahel, 1995). Since both surfactant classes are biotransformed several times faster by estuarine populations from the brackish layer than those from the underlying saline layer, it was suggested that the present surfactant-degrading bacteria were predominately of wastewater origin (Terzić *et al.*, 1992).

The effect of temperature on the biotransformation rate is rather pronounced. At the temperatures representative of summer (23 °C), the transformation rate of LAS is approximately 2 times faster than under winter temperature (14 °C) conditions. This effect is even more pronounced for NPnEO, mainly because of its very slow biotransformation under winter temperature conditions. The biotransformation is particularly inhibited at low temperatures in the saline layer, resulting in very long half-lives in that layer.

Table 1. Overall biotransformation rate constants for nonylphenol polyethoxylates (NPnEO) and linear alkylbenzene sulphonates (LAS) in brackish and saline compartments of the Sibenik Harbour.

Estuarine compartment	Temperature (°C)	NPnEO	LAS
		Rate constant (day <sup>-1</sup> )	Rate constant (day <sup>-1</sup> )
Brackish	13-14	0.02-0.03	0.161
	22.5-23	0.17-0.28	0.247
Saline	13-14	0.01-0.02	0.020
	22.5-23	0.02-0.05	0.156

#### ASSESSMENT OF ELIMINATION MECHANISMS AND RESIDENCE TIMES IN THE ESTUARY AND POSSIBLE IMPACT ON COASTAL WATERS

To assess the elimination and residence time of NPnEO surfactants, a simple calculation has been performed assuming that biodegradation and transport to the bottom sediment are the main elimination mechanisms. Taking into account a rather low concentration of aromatic surfactants in the surface sediments (<5 mg/kg), the transfer of surfactants to estuarine sediments was calculated to be less than 1 % of the total amount that enters the Sibenik Harbour. Therefore, this mechanism is not expected to be dominant for the removal of aromatic surfactants from the water column. The reason is, most probably, the hydrographic structure of the stratified estuary, which prevents an efficient transport of sediments to the bottom. Consequently, biotransformation should be considered the most important removal mechanism. The efficiency of elimination of aromatic surfactants by biotransformation was estimated using the one-box steady-state estuarine model proposed by Morris (1990). The original expression was modified to suit an irreversible first-order reaction as follows:

$$\text{Biodegradation (\%)} = kT / (1+kT),$$

where *k* and *T* represent the biotransformation rate constants and hydrodynamic residence time (flushing time), respectively. The average residence time of water in the brackish layer of the Sibenik Harbour was estimated at 1.2 days, while the residence time of the saline layer is much longer and varies from 6.2 days in winter to 18 days in summer (Legovic, 1991). Taking into account the overall biotransformation rates given in Table 1, it was estimated that the expected removal rate of NPnEO in the brackish layer ranges between 10 % in winter and 25 % in summer. In the saline layer, the elimination of NPnEO in winter is rather restricted (6%), but reaches 47 %

in summer. Most probable explanation for the incomplete elimination of aromatic surfactants in the brackish layer is a very short flushing time of that estuarine compartment, which does not provide enough time for the action of active bacterial populations. On the other hand, biotransformation kinetics of surfactants in the saline water layer is very slow, particularly in winter.

Based on this estimate, it is apparent that most of the surfactant residues entering the estuary are discharged into the coastal waters, though in a very dilute form. Results of a very recent screening of alkylphenolic compounds in Croatian coastal waters are shown in Table 2. As can be seen, the concentrations were found in the ng/L range and even the total concentrations never exceeded 1  $\mu\text{g/L}$ . The most abundant individual class are alkylphenoxy carboxylic acids (NP1EC and NP2EC).

Table 2. Determination of endocrine-disrupting alkylphenolic compounds in Croatian coastal waters (October 2003; concentrations in ng/L).

Location	NP1EO	NP2EO	OP	NP	BPA	OP1EC	NP1EC	NP2EC
Fazana	<30	15	<10	20	10	<10	20	50
Pula	<30	56	<10	50	10	10	280	400
Rijeka	<30	67	<10	20	10	<10	120	230
Bakar	<30	<10	<10	<10	10	10	70	40
Srima	<30	14	<10	20	<10	<10	80	180
Vranjic	<30	<10	<10	<10	<10	<10	50	50
Stobrec	<30	15	<10	30	10	<10	50	40
Gruz	<30	<10	<10	<10	<10	<10	10	<10

## CONCLUSIONS

Endocrine-disrupting alkylphenolic compounds are ubiquitous contaminants in municipal wastewaters; consequently, they can be found in all estuarine and coastal waters. At present, it seems that their concentrations in the water column of Croatian coastal waters are rather low and should not pose a direct threat to the fish populations. However, special attention should be paid to estuarine boundaries, where the concentration of the most toxic metabolites can be an order of magnitude higher than that in the bulk of the water column. Since estuarine interfaces represent sites of intensive biological activity, enhanced exposure concentrations in these compartments can be of major environmental concern.