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Research Note / Araştırma Notu

Mutagenicity of Nonylphenol and Octylphenol Using *Salmonella* Mutation Assay

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Özet: *Nonilfenol ve Oktifenol'ün* Salmonella *mutasyon testi ile mutajenitesinin belirlenmesi.* Bu çalışmada, yüzey suyunda, denizde, yeraltı sularında ve sucul sedimentte yüksek seviyelerde bulunan, endüstri ve bazı evsel ürünlerde yoğun olarak kullanılan Nonilfenol (NP) ve Oktilfenol (OP)' ün mutajenik aktivitesi test edilmiştir. NP ve OP'nin toksisite ve östrojenik etkisine ait çok sayıda veri bulunmasına rağmen, bu kimyasalların mutajenik etkisi ile ilgi literatür az sayıdadır. Mutajenik etkinin belirlenmesi amacı ile NP 0.937- 4.685 µg/L aralığın da 6 farklı konsantrasyonda, OP ise 10- 160 µg/L aralığında 5 farklı konsantrasyonda test sistemi olarak seçilen *Salmonella thyphimirium* TA98 ve TA 100 suşları üzerinde (metabolik aktivasyonsuz) test edilmiştir. Sonuçlar revertant koloni sayılarına göre değerlendirildiğinde, tüm NP konsantrasyonlarının toksik olduğu buna rağmen mutajenik etkisinin olmadığı gözlenmiştir. Buna ek olarak OP'ün *Salmonella thyphimirium* TA98 suşu ile yapılan testlerde 40 µg /L ve *Salmonella thyphimirium* TA100 suşu ile yapılan testlerde ise 20 µg/L konsantrasyonları mutajenik bulunmuştur. Bu bulgular ışığında yapmış olduğumuz çalışma çevresel konsantrasyonlarda ki NP' nin toksik, OP' nin ise mutajenik etkiye sahip olması ile çevresel etkilerinin önemini vurgulamaktadır.

Anahtar Kelimeler: Alkilfenol ethoksilat, nonyilfenol, oktilfenol, *Salmonella thyphimirium,* mutajenisite.

Abstract: In this study, mutagenic ability of Nonylphenol (NP) and Octylphenol (OP) were tested considering these chemicals are widely used in industry and in some domestic products and they are found widely in surface water, sea water, ground water and aquatic sediments. Although abundant data are available on the toxicity and estrogenic effects of NP and OP, little is known about the mutagenicity of these chemicals. Six concentrations ranging from 0.937 to 4.685 µg/L of NP and five concentrations ranging from 10 to 160 µg/L of OP was tested in the His+ revertant assay with the use of *Salmonella thyphimirium* TA98 and TA 100 without metabolic activation. All NP concentrations showed non-mutagenic activity although; it had toxic characteristics exhibiting growth rate less than that of the spontaneous revertant colonies. Besides that, mutagenicity was observed at 40 µg/L for OP tested with TA98 strains and 20 µg/L for OP tested with TA100 strains of *Salmonella thyphimirium*. These findings will contribute to assess their environmental impacts showing the mutagenic effects of OP and toxic effects of NP on *S.typhirmirum* in environmentally relevant concentrations.

Key Words: Alkylphenol ethoxylat, nonylphenol, octylphenol, *Salmonella thyphimirium,* mutagenicity.

Introduction

Alkylphenols (APs) are high production volume man-made chemicals, used primarily to manufacture alkylphenol ethoxylates (APEs). APs and APEs are in use for over 50 years and are important to a number of industrial processes, such as pulp, paper and textile industries and coatings, agricultural pesticides, lube oils, fuels, metals and plastics (APERC, 2004).

The most important alkylphenols are nonylphenol (NP) and octyphenol (OP). They exist in different forms, or "isomers", and are used to make nonylphenol ethoxylates (NPEs) and octylphenol ethoxylates (OPEs). NPEs were used primarily as surfactants in detergents as well as to produce resins and plastics and as antioxidants and stabilizers in plastics. NPs are employed in lubricant oil, cosmetics, emulsifiers, plastics, latex paints, household and industrial detergents, paper and textile industries (TemaNord, 1996). In 1997, prior to the restrictions on the use of NPEs, production and total usage of NP in the EU was 73.500 tons and 78.500 tons respectively (ECB, 2002).

The main areas of use of octylphenol are as an intermediate in the production of phenol/formaldehyde resins (98% of use) and in the manufacture of octylphenol ethoxylates (2% use). Consequently, a similar proportion of the "nonylphenol" produced will actually be octylphenol (OSPAR, 2006a). OPs are used mainly to make phenolic resins, although following the restrictions on NP/NPEs it has been suggested that OPEs might be used in place of NPEs in some of their applications (EA, 2005). OPs are used in the electrical insulating varnishes, as a tackifier in rubber for tyres, in ethoxylated resins, recovery of oil in offshore processes, printing inks, pesticide formulations (as a dispersant), water-based paints, textile auxiliaries and emulsion polymerization (OSPAR, 2006b; Nimrod and Benson, 1996). Approximately 23.000 tones of OP (4-tert octylphenol) were consumed in the EU in 2001 (EA, 2005). NP and OP are not produced naturally, therefore environmental concentrations result from anthropogenic activity. These are generally discharged in large quantities to aquatic environments either directly from untreated effluent or indirectly from sewage treatment plants (Maguire, 1999; Colborn *et al.*, 1993)

According to research, approximately 300,000 tones of APs are produced per year, 60% of this amount reaches the aquatic environment and here converted in to the biodegradation products NP and OP, which are more toxic than AP itself (Schrenk-Bergt and Steinberg, 1998). The environmental concentrations of these chemicals have been determined by several researchers. In some studies carried out in rivers, NP concentrations in water were measured between 45-71 µg/L and 7.2-52 ng/L (Ahel *et al.*, 1994; OSPAR, 2006b). As in rivers, the presence of APs and their metabolites in oceans and saltwater originates from polluted rivers and direct sewage discharge. Marcomoni et al (1990) found the NP level in surface water of Italy's Venice Lagoon to be 0.15-13.7 mg/L, while in a study conducted in Canada the amount of OP in surface water was reported as 0.084µg/L (Bennie *et al.*,1997). In treatment plants for domestic waste in Michigan, Synder *et al.* (1999) had reported the concentrations of NP ranging between 0.017-37 µg/L, while OP concentrations varied between 37-332 µg/L. Therefore, there is considerable evidence indicating that OP over a range of concentrations (0.02-2 mg/L) is a biologically active contaminant. Indeed, there is reason for concern because OP has been found at significant concentrations in fresh water (<0.2 ng/L-0.5 µg/L), sediments (<0.010-1.8 µg/g dry weight), sewage treatment effluent (0.1-2.5 µg/L), and sludge (<0.005-12.1 µg/g dry weight) in North America. Waterborne OP levels of approximately 12 µg/L have been reported in UK Rivers and estuaries (Trudeau *et al.*, 2002). NP and OP, which are present in certain concentrations in surface waters and sediments, accumulated biologically in the aquatic environment and are estrogenic and very toxic, because they are used in large quantities (Naylor *et al.*, 1992; McLeese *et al.*, 1981). Apart from the direct toxicity of NP and OP, they also mimic hormones in vertebrate (Soto *et al.*, 1991). Kim *et al.*, (2006) had studied toxicological effects of NP and OP on early embryonic development in humans and reported that OP induce apoptosis in human Embryonic Stem cells (hES) of human embryo in a dose-dependent manner and that the Fas and FasL pathway is involved in this process There is a large body of evidence on endocrine disrupting and acute and chronic toxic properties of NP and OP on several organisms tested *in vivo* or *in vitro* bioassays (Arslan *et al.*,2007; Arslan Cakal and Parlak, 2007; Comber *et al.*,1993; Rasmussen *et al.*,2002; Jobling and Sumpter, 1993). To date there has not been any reports on mutagenic properties of alkyl phenol ethoxylates while many tests of their ability to cause genetic damage have been negative. White *et al.* (1994) has been reported that octylphenol ethoxylates and nonylphenol ethoxylates (both with 9 ethylene oxide units) have each caused genetic damage in one of the many types of tests used to screen for this kind of effect.

Detecting the mutagens are especially important owing to their capability of inducing cancer and their potential to damage the germ line, which may lead to negative changes in future generations (Czyż *et al.*, 2002; Kataoka *et al.*, 2000). The purpose of the bacterial reverse mutation assay is to evaluate a chemical's genotoxicity by measuring its ability to induce reverse mutations at selected in several bacterial strains. The assay commonly referred to as the Ames test; Ames *et al.*, (1975) has proven too reliable for identification of a large number of mutagenic and potentially carcinogenic substances. Recently, the Salmonella/microsome mutagenicity test has been fully developed and validated as a promising routine test method. The Salmonella/microsome mutagenicity assay or the Ames test has been widely used to the test chemicals for their mutagenic potential (Ames *et al.*, 1975). Salmonella assay easy, rapid and it has the ability to define the general molecular mechanism of action of mutagen. It is sensitive to a wide range of mutagenic and carcinogenic chemicals (Alexander, 1981; McCann and Ames, 1976; McCann *et al.,* 1975; Maron and Ames, 1983).

The aim of this study is to investigate mutagenicity of NP and OP with test strains *S.typhimurium* TA 98 and TA 100 which are the most widely used in mutagenicity assay in the field of genetic toxicology (Cerna *et al.,* 1991).

Material and Methods

Nutrient broth and bacto agar were purchased from Criterion (Hardy Diagnostics). Dimethylsulfoxide (DMSO), histidine, biotin and sodium azide were purchased from Merck. Ampiciline trihydrate was provided from Sigma. We used NP ($C_{15}H_{24}O$; Aldrich) and OP [CH_3 $(CH_2)_7C_6H_4OH$; Aldrich] for tests. Stock solutions were prepared as 10,000 mg/L of NP and 10 mg/L of OP by dissolving the chemicals in dimethylsulphoxide (DMSO) (Sigma, St. Louis, Mo.; Cat. No: 67-68-5). Seven test concentrations of NP (0.937, 1.874, 2.811, 3.478, 4.685, 9.37 µg/L) and six test concentrations of OP (10, 20, 40, 80 and 160 µg/L) were made through dilution of the stock solutions; these were used to determine mutagenic effects of the two chemicals. The controls consisted of an untreated negative control, and a positive control (NaN_3 and Mitomycin C).

Salmonella mutagenicity tests were performed using the standard plate incorporation method (Maron and Ames, 1983) with the TA98 and TA100 strains of *Salmonella typhimurium*, without S9-derived metabolic activation. Several concentrations of NP and OP were added to 100 µL of an overnight culture of bacteria and 200 µL of melted agar containing 0.5mM histidine and biotin. The molten top agar was then poured onto a minimal glucose agar base plate and incubated at 37°C for 2 days. NaN_3 (1.5 µg/plate) and Mitomycin-C (0.5 µg/plate) were used as positive controls. Each chemicals and controls were assayed in triplicate. Following incubation, the number of revertant colonies (His^- revertants) was counted (Maron and Ames, 1983).

In this study, the sample was considered to be mutagenic when the number of revertant colonies in the test plates was doubled the number of revertants in solvent control (Kutlu *et al.*, 2004). The toxicity of the samples to the bacteria was evaluated on the basis of significantly reduced number of

revertants compared to solvent control (Zeytinoglu *et al.*, 2000). The results were given by means of nine plates from three replicates and ± Standard deviation values.

Results

The aim of the present study was to investigate the mutagenic potential of Nonylphenol (NP) and Octylphenol (OP) using Salmonella mutagenicity test (Ames test). Mutagenicity of the NP and OP as studied at different concentrations was evaluated according to the significance in the linear portion of the dose-response curve. Dose-related increase in the number of the revertants was considered as the evidence of mutagenicity (Mortelmans and Zeiger, 2000; Vargas *et al.*, 2001).

The findings of the *in vitro* Ames Salmonella mutagenicity test (without metabolic activation) of NP are presented in Table 1. In NP samples, the numbers of revertants of TA98 and TA100 strain were (significantly $p<0.05$) decreased comparing to negative controls at all tested concentrations. As the revertant colonies of the both TA98 and TA100 strains did not reach to double in number of negative control, it was approved that these concentrations of NP have not mutagenic character.

When the OP results were compared to the negative control of revertant colonies for strain TA 98 a significant increase was observed at 40 and 80 µg/L treatment groups for OP. According to classifications of numbers of revertants (Kotelevtsev and Ludmilla, 1995) in may be inferred that these concentrations of OP gave mutagenic responses and evaluated as strong mutagen (SM). The toxic effect was also observed with the decreased number of revertants than negative control at the higher concentrations (160 µg/L) (Table 1).

Table 1. Mutagenicity analysis of Nonylphenol and Octylphenol using *S. typhimurium* assay with TA98 and TA 100 strain in the absence of metabolic activation AMC (Ames Mutagenicity Criteria). (*) ($p<0.005$). Mitomycin-C (0.5 µg/plate): No growth.

Chemicals (µg/L)	TA98	AMC	TA100	AMC
Nonylphenol (NP)				
Negative Control (DMSO)	41 ±1.52		138.7±6.3	
Spontaneous	43±2		166±142.7	
0.937	38.67±6.2	NM	137±4.16	NM
1.874	35.33±3.8	NM	140±5	NM
2.811	57.67±2.7	NM	164.3±5.3	NM
3.478	43.33±2.6	NM	150±1	NM
4.685	40.7±0.9	NM	149±0	NM
9.37	34.3±0.9	NM	148±0	NM
Octylphenol (OP)				
Negative Control	41±1.52		135.7±5.5	
Spontaneous	43±2		166±12.7	
10	42.67±3.38	NM	141±1.7	NM
20	39±1.15	NM	1500±0*	VSM
40	1300±57.7*	VSM	3.33±2.8	toxic
80	398±47.9*	SM	0±0*	toxic
160	0±0	toxic	0±0*	toxic

**A-Cr: Ames criteria, SM: strong mutagen; M: moderate mutagen; WM: weak mutagen; NM: no mutagen*

On the other hand, the highest number of revertants (1500) were observed for TA 100 strain at the 20 µg/L for Op exposure and classified as VSM. The higher concentrations (40, 80 and 160 µg-OP/L) had caused the toxic effect and the numbers of revertants were decreased to zero (Table 1).

Discussion

Damage of DNA by environmental mutagens may be the main cause of death and disability in advanced societies as well as in wild life. It has been believed that this damage, accumulating during organism's lifetime, initiates most cancer and genetic defects. The suggested solution is prevention to identifying environmental mutagens and minimizing living thing's exposures. Rapid, accurate, *in vitro* tests, such as Salmonella/microsome test, should play a crucial role in realizing this goal (McCann and Ames, 1976).

According to AMES criteria, all tested concentrations of NP were not found to be mutagenic while 20 and 40 µg/L of OP was found to be mutagenic. Kubo *et al.* (2002) have studied mutagenicity of 255 environmental chemicals and they are not found mutagenicity in same concentrations of NP. Thus, results of their study were similar to those of the present study.

There are some studies showing the sensivity of TA 98 strain of *S. typhimurium* to be more resistant against to the toxic materials than TA100 strain (Soto *et al.*, 1991). Our findings supported to this result with the decreased revertant numbers of TA100 strain than TA98 exposed to same OP concentrations.

Besides that some of investigators had reported that although NP and OP caused a genetic damage, these chemicals were not found mutagenic (White *et al.*, 1994; Cox, 1996). The results of our study showed that octylphenol has very strong mutagenicity on TA98 strain in the concentrations of 80 µg/L of OP while very strong mutagenicity on TA 100 strain the concentrations of 20 µg/L for same compound. The environmental concentrations of NP and OP were measured in wide range in different aquatic ecosystems. Some of them are close to our experimental concentrations but some of them much lower or higher.

NP and OP is persistent organic chemicals which may be accumulated by organisms by the either way of uptake from environment and bio-concentration factor (BCF) of nonylphenol ethoxylates as high as 10.000 has been measured in algae, 3-1300 in fish (Ahel *et al.*, 1993; Ekelund *et al.*, 1990). Considering this findings, the most important conclusion is detection of mutagenity of OP in the concentrations used in this study which are environmentally relevant. Besides that, this study showed that OP has potent to be frame shift and base-pair mutagens.

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