DETERMINATION OF ENDOCRINE DISRUPTING COMPOUNDS IN PAPER MILL WASTEWATERS, COMPARISON OF DIFFERENT WASTEWATER TREATMENTS FOR THEIR REMOVAL AND POTENTIAL GENOTOXIC ACTIVITY OF PAPER MILL WASTEWATERS

DISSertation

Damjan BALABANIČ

Mentors: Prof. Dr. Metka Filipič
Dr. Katja Benčina, uni. dipl. kem.

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LIST OF CONTENTS

ABBREVIATIONS ........................................................................................................... ix

1. INTRODUCTION ........................................................................................................... 1
   1.1 Endocrine disrupting compounds ........................................................................ 2
   1.2 Genotoxic compounds ....................................................................................... 3
   1.3 Determination of endocrine disrupting compounds .......................................... 5
      1.3.1 Chemically determination of endocrine disrupting compounds .......... 5
   1.4 Biological methods for the evaluation of mutagenic/genotoxic activity of paper mill wastewaters ........................................................................................................ 6
      1.4.1 Bacterial assays ......................................................................................... 6
      1.4.2 Eucaryotic assays ..................................................................................... 8
   1.5 Different treatment procedures to degrade and/or remove selected endocrine disrupting compounds from wastewaters ......................................................... 10

2. RESEARCH GOALS ...................................................................................................... 13

3. MATERIALS AND METHODS .................................................................................... 15
   3.1. Selection criteria of investigated endocrine disrupting compounds .............. 15
   3.2 Determination of selected endocrine disrupting compounds in two Slovenian paper mill wastewaters ...................................................................................... 16
      3.2.1 Sample locations, collection and preservation for chemical analysis .... 16
      3.2.2. Optimization of chemical analysis for determination of endocrine disrupting compounds present in paper mill wastewaters ........................................ 17
   3.3 Determination of mutagenic/genotoxic activity in two Slovenian paper mill wastewaters ............................................................................................................ 20
      3.3.1 Mutagenic/genotoxic activity ..................................................................... 20
   3.5 Different treatment procedures to degrade and/or remove selected endocrine disrupting compounds from paper mill wastewaters ........................................... 25
      3.5.1 Chemicals .................................................................................................... 25
      3.5.2 Sample collection ...................................................................................... 25
      2.5.3 Analysis of chemical oxygen demand ...................................................... 26
   3.5.4 Analysis of selected endocrine disrupting compounds ................................ 26
   3.5.5 Paper mill wastewater treatments from pilot plants ...................................... 27
   3.5.6 Paper mill wastewater treatments with advanced oxidation processes .......... 29
   3.5.7 Removal efficiency calculations .................................................................... 34

4. RESULTS AND DISCUSSION ...................................................................................... 35
   4.1 Determination of selected endocrine disrupting compounds in two Slovenian paper mill wastewaters ...................................................................................... 35
   4.2 Determination of mutagenic/genotoxic activity in two Slovenian paper mill wastewaters ........................................................................................................ 39
      4.2.1 Determination of selected endocrine disrupting compounds .......... 39
      4.2.2 Mutagenic/genotoxic activity .................................................................. 44
   4.3 Potential mutagenic/genotoxic activity of selected pure endocrine disrupting compounds ............................................................................................................ 62
      4.3.1 Bacterial assays ...................................................................................... 63
4.3.2 Human hepatoma HepG2 cells ................................................................. 65
4.4 Different treatment procedures to degrade and/or remove selected endocrine disrupting compounds from paper mill wastewaters ............................................. 73
  4.4.1 Removal of endocrine disrupting compounds by biological and membrane treatments ........................................................................................................ 73
  4.4.2 Laboratory scale advanced oxidation processes ....................................... 77
  4.4.3 Costs of investigated wastewater treatment processes ............................ 86

5. CONCLUSIONS ........................................................................................................ 89

6. SUMMARY ............................................................................................................. 91

POVZETEK ............................................................................................................. 94

7. REFERENCES ....................................................................................................... 97

ACKNOWLEDGEMENTS ..................................................................................... 114
LIST OF TABLES

Table 1: Sampling locations at investigated paper mills.......................................................... 17

Table 2: Pilot plants used and treatments applied................................................................. 26

Table 3: Average concentrations with ± SD of selected EDCs [dimethyl phthalate (DMP),
diethyl phthalate (DEP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), bis(2-
ethylhexyl) phthalate (BEHP), bisphenol-A (BPA), and nonylphenol (NP)] in raw (sample
location 1A and 1B) and biologically treated paper mill wastewaters (sample location 2A and
3B) according to the paper mills......................................................................................... 38

Table 4: The effect of paper mill A wastewater on the induction of SOS response in
Salmonella typhimurium TA1535/pSK1002........................................................................... 45

Table 5: The effect of paper mill B wastewater on the induction of SOS response in
Salmonella typhimurium TA1535/pSK1002........................................................................... 46

Table 6: Concentrations and molarity of selected compounds [dimethyl phthalate (DMP),
diethyl phthalate (DEP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), bis(2-
ethylhexyl) phthalate (BEHP), bisphenol-A (BPA), and nonylphenol (NP)]...................... 62

Table 7: The effect of dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate
(DBP), benzyl butyl phthalate (BBP), bis(2-ethylhexyl) phthalate (BEHP), bisphenol A
(BPA) and nonylphenol (NP) on the induction of SOS response in Salmonella typhimurium
TA1535 pSK1002. .................................................................................................................. 64

Table 8: Economical cost assessment of tested aerobic and anaerobic biological treatment and
membrane filtrations. ............................................................................................................... 87

Table 9: Economical cost assessment of tested advanced oxidation processes at laboratory
scale (Fenton process: [H₂O₂] / [Fe²⁺] = 1.5, [H₂O₂] / COD = 2.125; Photo-Fenton: [H₂O₂] /
[Fe²⁺] = 32 [H₂O₂] / COD = 2.125; Photocatalysis: [TiO₂] = 10 g/L, time=120 minutes; O₃:
time = 180 minutes; O₃ / H₂O₂: [H₂O₂ ] = 50 mM, time = 180 minutes). ............................... 87
LIST OF FIGURES

Figure 1: Chemical structure of endocrine disrupting compounds (EDCs) determined in the investigated paper mill wastewaters................................................................. 15

Figure 2: Sheme of pilot plant A. DAFs: dissolved air flotation units, UF: ultrafiltration, RO: reverse osmosis. .......................................................... 27

Figure 3: Sheme of pilot plant B. DAFs: dissolved air flotation units, MBR: membrane bioreactor, RO: reverse osmosis. .......................................................... 28

Figure 4: Sheme of lab equipment for UV irradiation.................................................. 32

Figure 5: Sheme of lab equipment for ozone treatment ............................................. 33

Figure 6: Average (± SD) for dimethyl phthalate, diethyl phthalate, dibutyl phthalate, benzyl butyl phthalate, bis(2-ethylhexyl) phthalate, bisphenol A and nonylphenol in paper mill A. . 41

Figure 7: Average (± SD) for dimethyl phthalate, diethyl phthalate, dibutyl phthalate, benzyl butyl phthalate, bis(2-ethylhexyl) phthalate, bisphenol A and nonylphenol in paper mill B. . 43

Figure 8: The mutagenic effect of paper mill B wastewater in Ames MPF™ 98/100 Aqua assay with Salmonella typhimurium TA100 strain. ............................................... 49

Figure 9: The level of DNA damage induced by untreated paper mill effluents (sample location 1B). The HepG2 cells were exposed to the 0, 10, 20 and 30 vol.% of water samples for 24 h. DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25th and 75th percentiles, the mean value is a solid line through the box. .......................................................... 52

Figure 10: The level of DNA damage induced by anaerobically treated paper mill effluents (sample location 2B). The HepG2 cells were exposed to the 0, 10, 20 and 30 vol.% of water samples for 24 h. DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25th and 75th percentiles, the mean value is a solid line through the box. .......................................................... 54

Figure 11: The level of DNA damage induced by aerobically treated paper mill effluents (sample location 3B). The HepG2 cells were exposed to the 0, 10, 20 and 30 vol.% of water samples for 24 h. DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25th and 75th percentiles, the mean value is a solid line through the box. .......................................................... 56
Figure 12: The level of DNA damage induced by surface water upstream of the discharge of effluents to the river (sample location 4B). The HepG2 cells were exposed to the 0, 10, 20 and 30 vol.% of water samples for 24 h. DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25th and 75th percentiles, the mean value is a solid line through the box. 

Figure 13: The level of DNA damage induced by surface water downstream of the discharge of effluents to the river (sample location 5B). The HepG2 cells were exposed to the 0, 10, 20 and 30 vol.% of water samples for 24 h. DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25th and 75th percentiles, the mean value is a solid line through the box. 

Figure 14: The level of DNA damage induced by untreated and treated paper mill effluents and artificially prepared mixtures of EDCs detected in corresponding water samples. The HepG2 cells were exposed to 30 vol.% of water samples and artificially prepared combination of EDCs with the same composition as real samples for 24 h. DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25th and 75th percentiles, the mean value is a solid line through the box. 

Figure 15: The level of DNA damage induced by selected EDCs. The HepG2 cells were exposed to the different concentrations (0, 1, 10, 100 and 1000 µg/L) of selected EDCs for 4 h (A, C, E, G, I, K and M) and 24 h (B, D, F, H, J, L and N). DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25th and 75th percentiles, the mean value is a solid line through the box. 

Figure 16: Removal efficiencies of chemical oxygen demand and investigated endocrine-disrupting compounds for different treatments in pilot plant A applied with standard deviations. 

Figure 17: Removal efficiencies of chemical oxygen demand and investigated endocrine-disrupting compounds for different treatments in pilot plant B applied with standard deviations. 

Figure 18: Removal efficiencies of chemical oxygen demand and investigated endocrine-disrupting compounds (A) and time intervals (B) for Fenton reaction applied with standard deviations. 

Figure 19: Removal efficiencies of investigated endocrine-disrupting compounds (A), chemical oxygen demand (B) and time intervals (C) for photo-Fenton reaction applied with standard deviations.
Figure 20: Removal efficiencies of investigated endocrine-disrupting compounds, chemical oxygen demand (A) and time intervals (B) for photocatalysis with TiO$_2$ reagent applied with standard deviations.

Figure 21: Removal efficiencies of investigated endocrine-disrupting compounds, chemical oxygen demand (A) and time intervals (B) for ozonation applied with standard deviations.
ABBREVIATIONS

AFB1 – aflatoxin B₁
AOP – advanced oxidation process
BaP – benzo [a] pyrene
BBP – benzyl butyl phthalate
BEHP – bis(2-ethylhexyl) phthalate
BPA – bisphenol A
BSTFA – N,O-bis(trimethyl-silyl)trifluoroacetamide
COD – chemical oxygen demand
DAF – dissolved air flotation
DBP – dibutyl phthalate
DEP – diethyl phthalate
DMP – dimethyl phthalate
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
EDC – endocrine disrupting compound
EDTA – ethylenediaminetetraacetic acid
EtBr – ethidium bromide
GC – gas chromatography
IR – induction ratio
logK_{ow} – octanol/water partition coefficient
LC – liquid chromatography
LMP – low melting point
MBR – membrane bioreactor
MLTSS – mixed liquid total suspended solids
MNNG – 1-Methyl-3-nitro-1-nitrosoguanidine
MS – mass spectrometry
MTBSTFS – N,O-bis(trimethylsilyl) trifluoroacetamide
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMP – normal melting point
NP – nonylphenol
OD – optical density
ONPG – *ortho*-Nitrophenyl-β-galactoside
PAH – polycyclic aromatic hydrocarbon
PCB – polychlorinated bisphenyl
PCP – pentachlorophenol
PFBr – pentafluorobenzyl
RO – reverse osmosis
SD – standard deviation
SPE – solid phase extraction
TBS – tributylsilyl
TGA – tryptone, glucose and ampicillin
TMS – trimethylsilyl
UF – ultrafiltration
WWTP – wastewater treatment plant
1. INTRODUCTION

The paper industry employs large amounts of water, and produces equally large amounts of wastewater, which constitutes one of the major sources of aquatic pollution. The additives (such as surfactants, glues, biocides and bleaching agents) and raw material (wood extractives) used in the paper production process, may produce highly toxic and refractory compounds, some of them are potentially mutagenic (Rigol et al. 2004; Wong et al. 2006). The paper mill effluents can cause slime growth, thermal impacts, scum formation, color problems and loss of aesthetic beauty in the environment. They can also increase the amount of toxic substances in natural water bodies, causing death to zooplankton and fish, as well as profoundly affecting the terrestrial ecosystem (Pokhrel and Viraraghavan 2004). Some raw materials used for paper production contain phthalates, which function as softeners in additives, glues and printing inks. Polycyclic aromatic hydrocarbon (PAHs) may also be found in printing inks, alkylphenols are constituents of widely used defoamers, cleaners and emulsifiers, while pentachlorophenol (PCP) is a major component of some biocides used in paper making industry (Wang et al. 2005; Kimura et al. 2007; Yu et al. 2007; Balabanič and Krivograd Klemenčič 2011). Since these compounds, when released into the environment may affect aquatic organisms or even enter human food chain through drinking water, it is of most importance to remove them from industrial wastewaters. According to the EU Water Framework Directive (2000/60/EC) and Directive (2008/105/EC) all industrial water pollution sources will have to be regularly analysed for the content of numerous compounds which are toxic, genotoxic, bioaccumulative or act as endocrine disruptors (Hamm et al. 2005a, 2005b). Consequently, individual producers will be obliged to reduce the impact of their discharges in order to fulfil the requirements of the directive.

Endocrine disrupting compounds (EDCs) and genotoxic compounds can cause adverse effects at very low concentrations and show their effects with the time delay (Purdom et al. 1994; Birkett 2003). The concentrations of EDCs and genotoxic compounds in wastewaters are often below the detection limit of chemical analytical methods (Birkett 2003; Petrovic et al. 2004). Human exposure to EDCs and genotoxic compounds may occur through several routes, including ingestion of food and water, inhalation of air and absorption through the skin. For most of these compounds, the major source of exposure is food.
Study from Schwarzenbach et al. (2006) shows that many environmental contaminants are of emerging concern because of their intrinsic biological activity, which may cause adverse effects, particularly at chronic exposure.

The conventional wastewater treatment processes (coagulation, sedimentation and filtration) are not specifically designed to degrade traces of these organic contaminants (Pokhrel and Viraraghavan 2004; Vethaak et al. 2005; Balabanič and Krivograd Klemenčič 2011), therefore it is essential that future research focuses on the development of appropriate treatment methods that can be integrated into water and wastewater treatment facilities to prevent the release of EDCs and other genotoxic contaminants into the natural water bodies. These organic contaminants have been found in sediments, wastewaters, surface waters, groundwaters and drinking waters (Snyder et al. 2003; Petrovic et al. 2004; Vethaak et al. 2005, Fernandez et al. 2007).

1.1 Endocrine disrupting compounds

In the last decades it has been reported that certain chemicals called EDCs may cause disruption of the endocrine system and can affect reproduction and development of exposed aquatic organisms, wildlife and also humans (Safe 2000; Baskin et al. 2001; Balabanič et al. 2011). An endocrine disrupter is an exogenous agent that interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour (US EPA 1997). EDCs include different groups of natural and synthetic compounds such as steroids (ethinyl estradiol, 17β-estradiol, estrone, mestranol and diethylstilbenestrol), alkylphenols (nonylphenol, nonylphenol ethoxylate, octylphenol and octylphenol ethoxylate), polyaromatic compounds (polychlorinated biphenyls (PCBs), PAHs and brominated flame retardants), organic oxygen compounds (bisphenol A and phthalates), pesticides (atrazine, chlordane, demeton-S-methyl, dichlorvos, dieldrin, dimethoate, endosulfan, hexachlorobenzene, lindane, linuron, PCP, permethrin, simazine and trifluralin) and others.
(tributyltin, dioxins and furans) as well as certain heavy metals (As, Cd, Pb and Hg) (Safe 2000; Baskin et al. 2001; Birkett 2003; Fisher et al. 2003; Al-Saleh et al. 2008; Balabanić et al. 2011).

Many EDCs are organochlorine substances, which mean that they contain chemically bound carbon and chlorine. This binding is strong and resistant to biochemical and physical degradation. The organochlorines have a long half-life and they accumulate in the environment as a persistent organic pollutant (Fisher 1999; Muir et al. 1999). Humans and most wildlife do not have biochemical pathways to detoxify or excrete these chemicals, so they tend to accumulate. The log\(K_{ow}\) (octanol/water partition coefficient) values for most EDCs indicate a high degree of lipophilicity (Birkett 2003). The studies showed that these compounds can mimic the natural hormones of animals and exposure has been linked to physiological effects in animals (Snyder et al. 2003). Environmentally detrimental chemicals with endocrine activity affect also human health and many of them have been shown to be genotoxic (Fernandez et al. 1998; Birkett 2003; Iso et al. 2006; Mocarelli et al. 2008; Balabanić et al. 2011). EDCs can produce testicular and prostate cancer (Carlsen et al. 1995; Birkett 2003; Fisher et al. 2003), cryptorchidism (McIntyre et al. 2001; Fisher et al. 2003), hypospadias (Baskin et al. 2001; McIntyre et al. 2001), female and male breast cancer (Fernandez et al. 1998; Toppari and Skakkebaek 2000), decreasing sperm counts and quality (Sharpe and Skakkebaek 1993; Carlsen et al. 1995; Fisher et al. 2003), endometriosis (Nicolopoulou-Stamati and Pitsos 2001), shortened menstrual cycle (Mendola et al. 1997), preterm birth (Perera et al. 2002), intruterine growth restriction (Perera et al. 2002) and many more.

### 1.2 Genotoxic compounds

Another important group of compounds relevant for the contamination of the environment are genotoxic compounds. Recent studies indicate that certain EDCs such as bisphenol A (BPA), diethylstilboestrol (DES) as well as PAHs and organochlorides are also genotoxic (Ali and Sreekrishnan 2001; Maria et al. 2003; Izzotti et al. 2009).
Mutagenic/genotoxic compounds, including carcinogens, whether known or unknown, are the components of complex environmental mixtures that can have adverse health effects on humans and indigenous biota (Dearfild et al. 2002). Mutagenicity is defined as a permanent change in the content or structure of the genetic material of an organism. A mutagenic hazard can be manifested as a heritable change resulting from germ-line mutations and/or somatic mutations leading to genetic disease and cancer or other chronic degenerative processes such as aging and coronary heart disease. These mutations may be expressed at the level of the individual gene or groups of genes, or at the level of the chromosomes. Although there are species differences in metabolism, deoxyribonucleic acid (DNA) repair, and other physiological processes affecting chemical mutagenesis, the universality of DNA and the genetic code provides a rationale for using various non-human test systems to predict the intrinsic mutagenicity of test chemicals. Additional support for the use of non-human systems is provided by the observation that chemicals causing genetic effects in one species or test system frequently cause similar effects in other species or systems (thus setting the stage for an assessment of possible mutagenic effects in humans) (Dearfild et al. 2002).

In the broad sense, genotoxicity pertains to all types of DNA damage (including mutagenicity), whereas mutagenicity pertains specifically to mutation induction at the gene and chromosome levels. Agents that interact with DNA and/or its enzymes or associated cellular components are designated genotoxic. Genotoxic effects include DNA adduct formation, DNA strand breaks, sister chromatid exchange and unscheduled DNA synthesis. It is recognized that some genetic changes will be lethal to the cell and therefore self-selecting for no phenotypic effect. However, other mutations, whether in somatic or germ cells, in genes or in chromosomes, may, or may not pose a human health hazard (Dearfild et al. 2002).

Genotoxic chemicals induce DNA damage and mutations, and chronic exposure to low doses of these chemicals may affect biodiversity, while in humans it may increase the risk for cancer development. There are many different types of genome damage, which arise through a diversity of mechanisms. Chromosomes might be broken (clastogenesis) or the information within the DNA sequence might become altered or rearranged (mutagenesis) (Moore and Bender 1993; Mayorov et al. 2005). Whole chromosomes might be mis-segregated
(aneugensis), and there can be interchange of sections of chromosomes, which can alter gene regulation (Maffei et al. 2000). These diverse outcomes are the consequence of different mechanistic interactions between the different classes of genotoxin and the exposed cell/organism. These vary from direct oxidative DNA damage to interference with the processes of DNA replication and repair (Kasprzak 1995; Hartwig et al. 2002; Henkler et al. 2010).

1.3 Determination of endocrine disrupting compounds

1.3.1 Chemically determination of endocrine disrupting compounds

Concentrations of EDCs in a complex matrix such as wastewater are around 1 ng/L (Petrovic et al. 2002). Wastewaters may contain many compounds that can interfere with analysis, that’s why determination of EDCs in wastewaters is an important analytical challenge (Lopez de Alda and Barcelo 2001). The challenge begins with the sampling of materials to be analysed and may be further subdivided into an extraction and sample preparation stage, which includes quantification step. The predominant method of EDCs quantification is either gas chromatography (GC) or liquid chromatography (LC) (Brossa et al. 2003; Zhao et al. 2009). The role of these two systems in analysis has been discussed by Grob (1991). GC is limited to the use of compounds that are thermally stable and volatile. The analytical methods proposed in the literature apply derivatization procedures before gas chromatography – mass spectrometry (GC–MS) analysis (Blau and Halket 1993). Different reagents have been used to derivatize EDCs, including pentafluorobenzyl (PFBr), \textit{N,O-}bis(trimethylsilyl) trifluoroacetamide (BSTFA) or \textit{N-}( tert- butyldimethylsilyl)-\textit{N-}methyl-trifluoro acetamide (MTBSTFA) that lead to the formation of trimethylsilyl (TMS) and tributylsilyl (TBS) derivatives (Ballesteros et al. 2006). These are often chosen because they are stable and allow the sensitivity to be improved (Blau and Halket 1993). The type of detector used with GC or LC systems depends on the compounds being determined.
1.4 Biological methods for the evaluation of mutagenic/genotoxic activity of paper mill wastewaters

The purpose of testing wastewater for genotoxicity is to identify whether wastewater contain genotoxic compounds. It is extremely difficult to quantify the risk associated with genotoxic compounds because they usually occur at concentrations too low to allow analytical determination. Using only physico-chemical analysis, it is impossible to predict the mutagenic/genotoxic properties of complex water samples, especially if synergistic or antagonistic effects between the components occur. On the contrary to chemical analysis, the biological assays give the response of the whole sample without knowing the composition of it. An alternative approach to characterise mutagenicity/genotoxicity of complex water samples is the use of biological tests, which produce a global response to the complex mixture of chemicals without any prior knowledge of the mixture composition or its chemical properties (Žegura et al. 2009). A number of biological assays have been introduced for determining the genotoxic potential of aquatic ecosystems. The biological assays such as SOS/umuC assay, Ames assay, alkaline DNA-elution assay, DNA alkaline unwinding assay, unscheduled DNA synthesis assay, micronucleus assay and comet assay are potentially useful for screening the genotoxicity of wastewaters (Filipič 1995; Buschini et al. 2004; Ohe et al. 2004; Chakraborty and Mukherjee 2009; Žegura et al. 2009). As already mentioned there are many genotoxicity assays, however, for detecting mutagenicity/genotoxicity in wastewater and surface water bacterial tests, particularly the Salmonella/microsomal mutagenicity test, has been the most widely used (Filipič 1995; Ohe et al. 2004).

1.4.1 Bacterial assays

The bacterial SOS/umuC and Ames assays have been predominantly applied to wastewater sample genotoxicity evaluation. All bacterial test methods have some common characteristics. Most tester strains contain mutations, which increase sensitivity to genotoxins. As bacteria do not possess the metabolic capacity of eucaryotes, the tests are usually performed in the absence and the presence of S9 liver homogenate to activate promutagens.
1.4.1.1 SOS/umuC assay

The SOS/umuC assay is based on the ability of genotoxins to induce expression of the umuC gene, one of the SOS genes responsible for “error-prone repair” (Hamer et al. 2000). Due to the participation of umuC in the mutagenic process leading to both, point- and frameshift-mutations, only one single bacterial strain is necessary to detect different kinds of mutagens. The tester strain *Salmonella typhimurium* TA1535/pSK1002 carries a fused umuC-lacZ gene, allowing for the monitoring of umuC expression by measuring β-galactosidase activity in a colorimetric assay. The SOS/umuC assay using a single tester strain detects many types of DNA damaging agents (Ohnishi et al. 1987), and can be used for the screening of environmental un-concentrated real samples.

1.4.1.2 Ames assay

Ames assay uses amino-acid requiring strains of *Salmonella typhimurium* (TA1535, TA1537, TA97, TA98 and TA100 strains) to detect point mutations, which involve substitution, addition or deletion of one or a few DNA base pairs (Ames et al. 1975; Maron and Ames 1983; Gatehouse et al. 1994). The principle of Ames assay is that it detects mutations which revert mutations present in the test strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain. The Ames assay is rapid, inexpensive and relatively easy to perform. This assay conducted *in vitro* generally require the use of an exogenous source of metabolic activation. Many of the test strains have several features that make them more sensitive for the detection of mutations, including responsive DNA sequences at the reversion sites, increased cell permeability to large molecules and elimination of DNA repair systems or enhancement of error-prone DNA repair processes. The specificity of the test strains can provide some useful information on the types of mutations that are induced by genotoxic agents. The Ames assay is commonly employed as an initial screen for genotoxic activity and in particular, for point mutation-inducing activity. An extensive data base has demonstrated that many chemicals that are positive in this test also exhibit mutagenic activity in other tests.
The novel Ames MPF™ 98/100 Aqua assay (Xenometrix) is designed to detect the mutagenic potential of un-concentrated water samples. The assay allows using several times more sample volume than with the conventional Ames assay for testing chemical compounds thanks to a concentrated Exposure Medium (Xenometrix 2010). The assay uses S. typhimurium TA98 and TA100 strains.

1.4.2 Eucaryotic assays

Tests with eukaryotic cells or organisms might be more relevant for human and ecological risk assessment than bacterial test systems, but generally they are much more time consuming. The most commonly used methods for assessing the genotoxic potential of substances on the basis of their principal genetic end-point are methods to investigate gene (point) mutation and methods to investigate chromosome aberrations. Several tests indicator have been developed using the integrity of DNA as an end-point of genotoxicity e.g. comet assay, alkaline DNA-eluation assay, DNA alkaline unwinding assay and unseduced DNA synthesis assay. The advantages of comet assay, relative to other genotoxicity assays are high sensitivity for detecting low levels of DNA damage, flexibility, low cost, ease of application, the need for relatively small amounts of test substance and a short period needed to complete the experiment (Tice et al. 2000).

Comet assay has applications in monitoring environmental contamination with genotoxins, nutrition toxicology, testing novel chemicals for genotoxicity, human biomonitoring and molecular epidemiology, free radical biology and fundamental research in DNA damage and repair (Collins 2004; Žegura and Filipič 2004). It was first developed by Östling and Johanson in 1984. The single cells embedded in agarose on a microscope slide are lysed by an alkaline buffer with ionic detergent at pH 10 to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Alkaline DNA denaturating conditions are added in order to detect, besides double strand breaks, single strand breaks, alkali-labile sites and repair-enzyme-mediated incisions. Cells with increased DNA damage display increased migration from the nuclear region towards the anode. The resulting comet-like structure is quantified by measuring the length of the tail and/or the tail moment observed by fluorescence
microscopy. The intensity of the comet tail relative to the head reflects the number of DNA breaks. The sensitivity and specificity of the assay are greatly enhanced if the nucleoids are incubated with bacterial repair endonucleases that recognize specific kinds of damage in the DNA and convert lesions to DNA breaks increasing the amount of DNA in the comet tail (Collins 2004).

To improve the sensitivity and specificity of the method, one can apply several modifications. The sensitivity can be increased also by using DNA repair inhibitors, which lead to the accumulation of DNA stand breaks. Specific classes of DNA adducts can be detected by incubating the lysed cells with lesion specific DNA repair enzymes (Žegura and Filipič 2004).

1.4.2.1 Test system with human hepatoma HepG2 cells

The majority of genotoxicity studies are carried out under in vitro conditions with bacterial and mammalian indicator cells which are devoid of enzymes involved in the activation of promutagens. In order to compensate for the lack of drug metabolizing enzyme systems, exogenous activation mixtures are added in these experiments. It is known for years that the limitations of the predictive value of short term in vitro assays are partly due to inadequate representation of the drug metabolism (Bigger et al. 1980; Tennant et al. 1987) and it is well documented that substantial differences in genotoxicity experiments are obtained when cellular or subcellular hepatic activation systems are used (Knasmüller et al. 1998).

This is especially important as most genotoxic compounds require metabolic activation to produce electrophilic species that can interact with the genetic material. The human hepatoma HepG2 cells have retained the activities of phase I enzymes which include the cytochromes P450 (functionalize compounds by oxidation, reduction or hydrolysis), whereas phase II enzymes carry out the conjugations of functionalized compounds with endogenous ligands (e.g., glucuronic acid, glutathione and sulfate), that play key roles in the activation and detoxification of DNA reactive carcinogens (Uhl et al. 2000). These enzymes are usually lost during in vitro cultivation (Uhl et al. 2000). Therefore, it is conceivable that genotoxicity assays with HepG2 cells reflect more adequately hazards of genotoxins than in vitro tests with
bacterial and mammalian cells, which require the addition of exogenous activation mixtures to mimic the metabolism of indirectly acting compounds (Uhl et al. 2000).

1.5 Different treatment procedures to degrade and/or remove selected endocrine disrupting compounds from wastewaters

Conventional wastewater treatment processes (coagulation, sedimentation and filtration) are not specifically designed to remove traces of organic contaminants in general (Kraigher et al. 2008; Balabanič and Krivograd Klemenčič 2011), so the latter are consequently consumed by aquatic organisms and thereby represent a hazard to the whole food chain. Investigations of appropriate treatment methods that can be integrated into water and wastewater treatment facilities to prevent the release of EDCs into the natural waters bodies are essential.

Activated sludge systems have been successfully applied to treat a wide variety of wastewaters; more than 90% of the municipal and industrial wastewater treatment plants (WWTPs) use this treatment type as an important part of their treatment train (Liu 2003). Numerous microorganisms, including bacteria, fungi and yeasts, predominantly aerobic microorganisms, are known for their ability to degrade hydrocarbons to carbon dioxide and water (Goronszy et al. 1995; Reginato et al. 1995; Suzuki et al. 1998; Van Hamme et al. 2003; Shokrollahzadeh et al. 2008). Biological treatment, particularly by activated sludge process, has been widely used for the degradation of organic compounds from paper mill wastewaters (Thompson et al. 2001; Pokhrel and Viraraghavan 2004; Balabanič et al. 2009; Balabanič and Krivograd Klemenčič 2011). Biodegradation is a dominant mechanism of phthalates, bisphenol A and alkylphenols degradation in waters (Staples et al. 1997; Soares et al. 2008; Zhao et al. 2008). Many studies on the biodegradation of phthalates, bisphenol A and alkylphenols under either aerobic or anaerobic condition have been reported (Staples et al. 1997; Soares et al. 2008; Zhao et al. 2008). The biodegradability of these compounds by anaerobic treatment is strongly dependent on the characteristics of the wastewater (Vidal and Diez 2005). The proportion of EDCs degradation by primary settling, aerating volatilization, chemical precipitation and sludge absorption is relatively small; the majority of EDCs
degradation from wastewater is regarded as biodegradation (Liu et al. 2009). Incomplete degradation of EDCs by existing biological WWTPs not only results from the fluctuation of EDCs levels in the influent, but also from the processes in biological WWTPs and as well from the operational conditions (Liu et al. 2009).

Furthermore, some studies have shown that membrane bioreactors (MBRs) could remove more than 80% of organic EDCs from wastewater (Wintgens et al. 2002). Compared to conventional wastewater treatment, a remarkable advantage of MBR is the high quality of effluent, including extremely low EDCs concentrations without chemical treatment. Removal of EDCs by membrane filtration technologies is not like biodegradation or chemical oxidation, as no by-products or metabolites are newly produced. Membrane filtration technologies, such as ultrafiltration (UF) and reverse osmosis (RO) have been shown as a promising alternative for removing micro-pollutants (Yoon et al. 2004; Yoon et al. 2006). RO enables almost complete removal of pollutants, but the high energy consumption is an important drawback to be considered. Comparing membrane types, the EDC rejection rate is the highest for RO, followed by nanofiltration, UF and finally microfiltration, as reported by Chang et al. (2003). Several studies have discovered that rejection efficiency of EDCs by membranes strongly depends on physico-chemical properties of these compounds, such as molecular weight, logKow, water solubility and electrostatic properties (Liu et al. 2009). Their removal by the membrane filtration technologies is mainly due to size exclusion, charge repulsion and adsorption (Liu et al. 2009).

Finally, advanced oxidation processes (AOPs) have been proved to be powerful in destroying most hazardous materials in water (Malato et al. 2002; Katsumata et al. 2004; Will et al. 2004; Esplugas et al. 2007; Gültekin and Ince 2007; Xu et al. 2008). AOPs can be broadly defined as aqueous phase oxidation methods based on the intermediacy of highly reactive species such as hydroxyl radicals in the mechanisms leading to the destruction of the target pollutant (Klavarioti et al. 2009). Unlike many other radicals, hydroxyl radical is non-selective and thus readily attacks a large group of organic chemicals to convert them to less complex and less harmful intermediate products (Klavarioti et al. 2009). At sufficient contact time and proper operation conditions, it is practically possible to mineralise the target pollutant to CO₂, which is the most stable end product of chemical oxidation (Gültekin and Ince 2007). Homogeneous
AOP employing hydrogen peroxide with UV irradiation has been found to be very effective in the degradation of EDCs (Al-Momani et al. 2004). In UV/H$_2$O$_2$ process the photolysis of hydrogen peroxide generates effective oxidizing species hydroxyl radical, which can oxidize a broad range of organic pollutants quickly and non-selectively (Alfano et al. 2001). Photocatalytic reactions via photo-generated holes of TiO$_2$ have been thoroughly investigated for water remediation during the last couple of decades. Among the photocatalytic oxidation materials, TiO$_2$ is a catalyst which is stable to photocorrosion and has low cost (Chung and Chen 2009). Under near UV irradiation, TiO$_2$ is photoactivated to form active oxygen species (-OH radicals) on surfaces of the crystal, and the radicals readily react with a wide range of organic moieties to ultimately produce CO$_2$. Several studies related to the use of TiO$_2$ on the degradation of EDCs have been reported (Nakashima et al. 2002; Tsai et al. 2009; Wang et al. 2009; Daskalaki et al. 2011). Ozone can be used for treatment of effluents from various industries (Baban et al. 2003). A major disadvantage of the ozonation is the relatively high cost of ozone generation coupled with the short half-life of ozone (Sevimli, 2005). Therefore, ozone needs to be generated at site. Some studies indicate that ozonation was highly efficient for degradation of organic pollutants and decolorization (Pokhrel and Viraraghavan 2004).
2. RESEARCH GOALS

The main goals of the research could be divided into three parts:

The first research goal was to determine the presence of seven endocrine disrupting compounds (EDCs) (dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), bis(2-ethylhexyl) phthalate (BEHP), bisphenol A (BPA) and nonylphenol (NP)) in the influents and effluents of paper mill wastewaters and associated surface waters of two Slovenian paper mills (paper mill A and paper mill B). Some of them: BEHP, BPA and NP are on the list of priority substances according to EU Water Framework Directive (2000/60/EC) and Directive (2008/105/EC), which will enter into force in 2015. DMP, DEP, DBP and BBP were chosen as it was well known from the literature that they could occur in the effluents of paper industry.

The second research goal was to assess genotoxic activity of paper mill wastewater and associated surface water samples in which DMP, DEP, DBP, BBP, BEHP, BPA and NP were determined. Based on the obtained results the assays were evaluated for their applicability as an integral tool in the evaluation of toxic potential of complex wastewaters prior to their release into the environment, as well as for the monitoring of surface and wastewater quality, providing the data useful for risk assessment. The comparison of the results obtained from bacterial and eukaryotic assays showed whether the bacterial assays, which were relatively inexpensive and the results obtained within some hours or days, were sensitive enough to be used in the monitoring of potentially mutagenic/genotoxic activity of un-concentrated paper mill wastewaters. We also determined the genotoxic activity of pure EDCs (DMP, DEP, DBP, BBP, BEHP, BPA and NP) and their artificial mixtures at concentrations (1–1000 μg/L) detected in the samples of both kind of paper mill wastewaters. The genotoxic activity was determined using combination of standard bacterial test – SOS/umuC test and comet assay with human hepatoma HepG2 cells. In the literature we could not find the genotoxicity data for all the target compounds detected in our paper mill wastewater samples. Therefore these results gave new information regarding to genotoxic activity of these EDCs. Based on the obtained results we were able to predict whether and to what extent the presence of EDCs
contributed to positive genotoxicity results in wastewater samples or was the genotoxicity predominantly due to the presence of other unknown contaminants in the wastewater samples.

The third research goal was to explore which wastewater treatment procedures were more efficient to degrade and/or remove selected EDCs from paper mill wastewaters applying the chemical analysis before and after various treatment procedures. Different treatment procedures: anaerobic and aerobic biological treatment, membrane bioreactor, ultrafiltration and reverse osmosis on pilot-scale, as well as Fenton and photo-Fenton reaction, photocatalysis with TiO$_2$ reagent and ozonation on lab-scale were compared. According to the efficiencies of different paper mill wastewater treatments, calculations of approximately costs were made and on this basis we could conclude, which method was more affordable and environmentally acceptable.
3. MATERIALS AND METHODS

3.1. Selection criteria of investigated endocrine disrupting compounds

The investigated compounds (DMP, DEP, DBP, BBP, BEHP, BPA and NP) (Figure 1) were chosen based on the requirements of the EU Water Framework Directive (2000/60/EC) and Directive (2008/105/EC), which will enter into force in 2015. BEHP, BPA and NP are already on the list of priority substances in EU Water Framework Directive (2000/60/EC) and Directive (2008/105/EC). DMP, DEP, DBP and BBP were chosen as it is well known from the literature that they can occur in the effluents of paper industry (Furhacker et al. 2000; Fukazawa et al. 2001; Hamm et al. 2005a, 2005b; Lattore et al. 2005; Carabias-Martinez et al. 2006; Kersten et al. 2006).

**Figure 1:** Chemical structure of endocrine disrupting compounds (EDCs) determined in the investigated paper mill wastewaters.
3.2 Determination of selected endocrine disrupting compounds in two Slovenian paper mill wastewaters

3.2.1 Sample locations, collection and preservation for chemical analysis

Samples were collected from untreated and biologically treated wastewaters of two Slovenian paper mills (paper mill A and paper mill B) and from associated surface waters 50 m upstream and 50 m downstream of both paper mill effluent discharges. Paper mill A (water consumption 286 m$^3$/h, wastewater amount 277 m$^3$/h) is manufacturing label papers and flexible packaging papers from fresh cellulose fibres and has on site aerobic biological wastewater treatment plant (BWTP) with retention time 48 h. Paper mill B (water consumption 287 m$^3$/h, wastewater amount 273 m$^3$/h) is manufacturing carton board from 100% recovered fibres and has on site combined aerobic-anaerobic BWTP with retention time 30 h. The samples were taken in the period from August 2008 to May 2009. Samples were collected in 2.5 L glass containers. Samples were taken at four sampling sites at paper mill A and at five sampling sites at paper mill B. The locations of the sampling sites are presented in Table 1. The locations at paper mill A are as follows: the first location (1A) was wastewater before aerobic treatment, the second location (2A) was wastewater after aerobic treatment, the third location (3A) was surface water upstream and the fourth location (4A) was surface water downstream of the discharge into the watercourse. The locations at paper mill B are as follows: the first location (1B) was wastewater before anaerobic treatment, the second location (2B) was wastewater before aerobic treatment (after anaerobic treatment), the third location (3B) was wastewater after aerobic treatment, the fourth location (4B) was surface water upstream and the fifth location (5B) was surface water downstream of the discharge into the surface waters (river) (Table 1).
Table 1: Sampling locations at investigated paper mills.

<table>
<thead>
<tr>
<th>Paper mill A</th>
<th>Paper mill B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>wastewater before aerobic treatment</td>
</tr>
<tr>
<td>2A</td>
<td>wastewater after aerobic treatment</td>
</tr>
<tr>
<td>3A</td>
<td>surface water upstream of the paper mill wastewater discharge into the surface water</td>
</tr>
<tr>
<td>4A</td>
<td>surface water downstream of the paper mill wastewater discharge into the surface water</td>
</tr>
<tr>
<td>5B</td>
<td>surface water downstream of the paper mill wastewater discharge into the surface water</td>
</tr>
</tbody>
</table>

After collecting, the samples were filtered (Albet nylon syringe filter, 0.45 μm), and formaldehyde (3%, v/v) was added to prevent changes in the composition due to biodegradation. Before the extraction the samples were stored in the dark at 4 °C (the time between collection and extraction was less than 24 h). For biological assays the samples were collected, filtered (Whatman cellulose acetate syringe filter, 0.2 μm) and storage at -80°C.

3.2.2. Optimization of chemical analysis for determination of endocrine disrupting compounds present in paper mill wastewaters

3.2.2.1 Chemicals and standards

All chemicals (DMP, DEP, DBP, BBP, BEHP, BPA and NP) were of analytical grade and supplied by Sigma-Aldrich (Steinheim, Germany). Primary stock solutions of DMP, DEP, DBP, BBP, BEHP, BPA and NP were prepared individually. Stock standard solution (100 mg/L) of each EDCs were prepared and stored in glass bottles at 4 °C until use. These solutions were used to spike the water samples.
3.2.2.2 Extraction of selected endocrine disrupting compounds

The first step was the choice of most effective method for organic compounds extraction from aqueous matrix. Three different types (Supelclean ENVI-18, Strata-X and Oasis HLB) of solid phase extraction (SPE) cartridges were tested. Supelclean ENVI-18 (500 mg/6 mL) cartridges were supplied by Sigma-Aldrich (Steinheim, Germany), Strata-X (200 mg/6 mL) cartridges were supplied by Phenomenex (Torrence, USA) and Oasis HLB (500 mg/6 mL) cartridges were supplied by Waters (Milford, USA). Each test was performed in five parallels. Average recoveries of analytes using the selected cartridges were as follows: for Supelclean ENVI-18 were in the range between 78 and 87 %, for Strata X were in the range between 73 and 85 % and for Oasis HLB were in the range between 95 and 103 %. Based on the testing of several types of SPE cartridges, Oasis HLB cartridge (6 mL) filed with 500 mg of poly(divinylbenzene-co-N-vinypyrrolidone) sorbent were chosen as the most efficient one.

SPE extraction was performed after the filtration (Albet nylon syringe filter 0.45 μm) and acidification of the samples. Optimization of the SPE extraction of DMP, DEP, DBP, BBP, BEHP, BPA and NP from the wastewater samples using Oasis HLB SPE cartridges was performed according to the Waters Oasis applications for GC-MS analysis of EDCs (Waters, 2008) with minor modifications.

For each sample the sorbent was conditioned with 2 mL methyl t-butyl ether, 2 mL methanol, and 2 mL deionized water. Subsequently, 1 L of wastewater sample was passed through Oasis HLB cartridge (6 mL) filled with 500 mg of poly(divinylbenzene-co-N-vinypyrrolidone) sorbent. After drying by vacuum pressure, the cartridge was rinsed with 2 mL methanol/water (40/60), 2 mL deionized water, and 2 mL methanol/ammonia/water (10/2/88). The sample was then eluted with 3 mL methanol/methyl t-butyl ether (10/90). After the evaporation of the methanol/methyl t-butyl ether solution, the extract was derivatized with BSTFA. All samples were done according this protocol.
3.2.2.3 Derivatization

Sample extract was derivatized in a test tube by the addition of BSTFA according to the (Ballesteros et al. 2006; Paraskevi and Voutsa 2008) with minor modifications. Briefly, 3 mL of the sample extract with internal standard solutions was evaporated to dryness under nitrogen gas flow. Then 100 μL of BSTFA was added, the test tube was closed and placed in a hot water bath (≈60 °C) for 15 min. 500 μL of an isooctane/acetone solution (99/1) was added and the test tube was then placed in an ultrasonic bath for 5 min. After derivatization, the extract was ready for injection into the GC-MS system.

3.2.2.4 Gas chromatography-mass spectrometry analysis

Analyses were conducted according to slightly modified standard method (APHA 2005). Briefly, an Agilent 7890 GC-MS system with autosampler was used for the determinations of DMP, DEP, DBP, BBP, BEHP, BPA and NP. The injector was operated in splitless mode. Analytes were separated on a DB5-MS column (30 m x 0.25 mm ID x 0.25 μm) using a 1 mL/min helium flow. The temperature program was as follows: initial temperature 50 ºC (4 min), heating rate 8 ºC/min, final temperature 270 ºC (5 min). Each sample was analyzed in five parallels, to allow for statistical analysis of the data. Calibration curves for each analyte were in the range between 0.5 and 500 µg/L. R^2 values were as follows: for dimethyl phthalate 0.996, for diethyl phthalate 0.997, for dibutyl phthalate 0.996, for benzyl butyl phthalate 0.998, for bis(2-ethylhexyl) phthalate 0.997, for bisphenol A 0.996 and for nonylphenol 0.997.

Individual phthalates, bisphenol A and nonylphenol were identified by characteristic MS spectra while their concentrations were calculated from the calibration curves of standard solutions.
3.3 Determination of mutagenic/genotoxic activity in two Slovenian paper mill wastewaters

Samples from paper mill A and paper mill B for chemical analysis and genotoxicity determination were taken at the same locations as for the determination of EDCs (described in Section 3.2.1) (Table 1). In this part of the study the sampling was repeated each day at the same time (morning) for seven days. All together 63 samples were taken from both paper mills (28 samples from paper mill A, and 35 samples from paper mill B). The samples from paper mill A were taken in September 2009, and samples from paper mill B were taken in January 2010. All the samples were analysed by GC-MS as described in Materials and methods (see Section 3.2.2).

3.3.1 Mutagenic/genotoxic activity

The mutagenicity/genotoxicity of paper mill wastewaters and associated surface waters was determined using the combination of standard bacterial test systems – SOS/umuC assay (ISO 2000), and bacterial assay for reverse mutations – Ames MPF™ 98/100 Aqua assay (Xenometrix 2010) and with the genotoxicity comet assay in the cell model with human hepatoma HepG2 cells. The potential mutagenic/genotoxic activities of pure and artificial mixtures of EDCs (DMP, DEP, DBP, BBP, BEHP, BPA and NP) were determined using the combination of bacterial test – SOS/umuC assay and human hepatoma cell line model (HepG2 cells) using comet assay. These chemicals were chosen as they occurred in wastewater samples from both Slovenian paper mills. The concentrations of pure EDCs and in artificial mixtures were in ranged from 1 to 1000 μg/L as these concentration were detected in researched samples.
3.3.1.1 Bacterial assays

3.3.1.1.1 SOS/umuC ASSAY

The SOS/umuC assay was performed according to Reifferscheid et al. (1991), with modifications described in the ISO standard ISO/CD 13829 (ISO 2000) for testing unconcentrated environmental samples.

Briefly, the overnight bacterial culture S. typhimurium TA1535/pSK 1002 was diluted 10 times with fresh tryptone, glucose and ampicillin (TGA) medium and incubated at 37 °C for 90 min with shaking (250 rpm) until the bacteria reached the exponential growth phase. The test was performed in triplicate on a 96 wells microtiter plate with the incubation mixture consisting of 180 μL un-concentrated water sample, 20 μL 10xTGA and 70 μL of bacterial culture or in the case of metabolic activation 180 μL un-concentrated water sample, 20 μL 10xTGA with cofactors and 70 μL of S9 bacterial culture mixture as described in the ISO standard. For pure EDCs the test was performed in triplicate on a 96 wells microtiter plate with the incubation mixture consisting of 10 μL of the sample, 90 μL of bacterial culture in TGA medium or in the case of metabolic activation 90 μL of bacterial culture in TGA medium with cofactors and S9 mixture. The microtiter plate was incubated at 37 °C for 2 h with shaking (250 rpm). The incubation mixture was then diluted 10 times with fresh TGA medium and incubated for further 2 h. The bacterial growth rate was determined by measuring absorbance at 600 nm. β-galactosidase activity was determined using ortho-Nitrophenyl-β-galactoside (ONPG) as a substrate after 20 min at 25 °C. Absorption was measured at 420 nm using a reference solution without bacteria. The bacterial growth rate was calculated using the following formula: \[ G = \frac{(\text{sample OD}_{600} - \text{blank OD}_{600})}{(\text{control OD}_{600} - \text{blank OD}_{600})} \]. A growth ratio less than 0.75 that represents 25% inhibition of biomass growth was consider to be indicative of water samples being cytotoxic.

β-galactosidase activity in relative units was calculate from the formula: \[ U = \frac{(\text{sample OD}_{420} - \text{blank OD}_{420})}{(\text{sample OD}_{600} - \text{blank OD}_{600})} \] and induction ratio (IR) according to the formula: \[ (1/G) \times \frac{(\text{sample OD}_{420} - \text{blank OD}_{420})}{(\text{control OD}_{420} - \text{blank OD}_{420})} \]. An induction ratio ≥1.5 was taken as the threshold at which the sample was considered as...
genotoxic (ISO, 2000). The standard deviation (SD) was calculated by error multiplication according to Gellert et al. (1968).

1-Methyl-3-nitro-1-nitrosoguanidine (MNNG; 6 μM) was used as the positive control without and aflatoxin B1 (AFB1; 2 μg/mL) with metabolic activation (S9).

3.3.1.1.2 AMES MPF™ 98/100 AQUA ASSAY

Ames MPF™ 98/100 Aqua assay is a modification of a classical Ames assay designed for measuring un-concentrated environmental water samples. The assay was performed according to manufacturer's protocol (Xenometrix 2010). In the assay Salmonella typhimurium TA98 and TA100 strains are used.

Approximately $10^7$ his-bacteria were exposed to undeluted water samples, as well as positive and a negative control for 90 minutes in medium containing sufficient histidine to support approximately two cell divisions. After 90 minutes, the exposure cultures were diluted with pH indicator medium lacking histidine, and distributed into 48 wells per sample. Within two days, cells that have undergone the reversion to histidine prototrophy were grown into colonies. Metabolism by the growing bacterial colony reduces the pH of the medium, changing the color of that well. A sample that yields a reproducible increase of greater than base line (BL) is classified as a mutagen (Xenometrix, 2000). Each sample was tested in triplicate, to allow for statistical analysis of the data. An increase in the number of revertant colonies upon exposure to sample relative to the negative control indicates that the sample is mutagenic in the Ames MPF™ 98/100 Aqua assay. The mutagenic potential of samples can be assessed directly or in the presence of liver S9 fractions. Undiluted samples that gave positive results were further tested in dilutions (1:1, 1:2 and 1:4) to confirmed their mutagenic activity. The mutagenic activity of the samples was calculated according to the MS Excel template provided by the Xenometrix (Xenometrix, 2010). Mixture of 2-nitrofluorene (2-NF; 0.1 μg mL$^{-1}$) and 4-nitroquinoline (4-NQO; 2 μg mL$^{-1}$) was used as a positive control without and 2-aminoanthracene (2-AA; 5 μg mL$^{-1}$) with metabolic activation, while distilled water was used as the negative control.
3.3.1.2 Human hepatoma HepG2 cells

Human hepatoma cell line (HepG2 cells) was used to measure genotoxic potential of water samples, pure DMP, DEP, DBP, BBP, BEHP, BPA and NP, and their artificial mixtures. HepG2 cells were grown in William’s medium E containing 15% foetal bovine serum, 2 mM L-glutamine and 100 U mL\(^{-1}\) penicillin/streptomycin at 37 °C in 5% CO\(_2\). The cells were used at passages between six and nine. In all experiments, the results of water sample treated cells were compared to a distilled water control.

3.3.1.2.1 CITOTOXICITY ASSAY (MTT ASSAY)

The cytotoxicity of the samples was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to Mosmann (1983), with minor modifications (Žegura et al. 2009). This assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of the intact mitochondria of living cells. HepG2 cells was seeded at a density of 8 x 10\(^3\) cells/well into 96-well microtiter plates in five replicates. After 24 h incubation at 37 °C to allow attachment of the cells, the growth medium was replaced with fresh medium containing 30 vol.% of water samples (in the case of pure EDCs tested, the growth medium was replaced with fresh medium containing different concentrations of pure EDCs [1, 10, 100 and 1000 µg/L]) and the cells were incubated for additional 24 h. After treatment, MTT was added at a final concentration of 0.5 mg/mL and the cells were further incubated for 3 h at 37 °C. The medium was removed and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The amount of formazan crystals directly correlates to the number of viable cells. The optical density (OD) was measured at 570 nm (reference filter 690 nm) using a spectrofluorimeter (Tecan, Genios). Benzo [a] pyrene (BaP) (75 µM) was used as a positive control. Cell survival (viability) was determined by comparing the OD of the wells containing cells treated with water samples to cells exposed to growth medium. A 30% reduction of the viability by the sample is consider a cytotoxic response (Žegura et al. 2009). Student’s t-test was used to compare the cell survival between distilled water control and groups exposed to water samples.
HepG2 cells were seeded in 12-well cell culture cluster plates, and left overnight to allow the attachment of cells. Then the growth medium was replaced with fresh medium containing 30 vol.% of water samples (in the case of pure EDCs tested, the growth medium was replaced with fresh medium containing different concentrations of pure EDCs [1, 10, 100 and 1000 µg/L]) and the cells were incubated for 24 h. BaP (30 µM) was used as a positive control. After the treatment, the cells were trypsinized and centrifuged at 800 rpm for 5 min and comet assay was performed.

Comet assay was performed according to Tice et al. (2000) with minor modifications described in Žegura and Filipič (2004). Briefly, 30 µL of cell suspension was mixed with 70 µL 1% low melting point (LMP) agarose and added to fully frost slides coated with 80 µL of 1% normal melting point (NMP) agarose. Subsequently, the cells were lysed (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA) Na₂, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h at 4 °C, then placed into an alkaline solution (300 mM NaOH, 1 mM EDTA Na₂, pH 13) for 20 min at 4 °C to allow DNA unwinding and electrophoresing for 20 min at 25 V (300 mA). Finally, the slides were neutralized in 0.4 M Tris buffer (pH 7.5) for 15 min, stained with ethidium bromide (EtBr) (5 µg/mL) and analysed using a fluorescence microscope (Nikon, Eclipse 800). Images of 50 randomly taken nuclei per experimental point were analysed with the image analysis software (Comet Assay IV, Perceptive Instruments, UK). For each sample, two independent experiments were performed. Samples that were statistically significant different from the control, were further tested in dilutions (30%, 20%, and 10%) to confirmed their genotoxic activity. A one-way analysis of variance (ANOVA, Kruskal-Wallis) was used to analyse the differences between treatments within each experiment. The Dunnett's test was used for multiple comparison versus the control; \( p < 0.05 \) was considered as statistically significant (*). Statistical analyses were done with GraphPad Prism 5 program.
3.5 Different treatment procedures to degrade and/or remove selected endocrine disrupting compounds from paper mill wastewaters

Wastewater from a newsprint paper mill was used in the experiments. This mill uses 100% recovered paper as raw material and tap water coming from the regional water utility as fresh water.

3.5.1 Chemicals

TiO₂ was supplied by Evonik (Essen, Germany). All other chemicals were of analytical grade and supplied by Panreac (Barcelona, Spain).

3.5.2 Sample collection

Samples were collected from a 100% recovered paper mill and from two pilot plants located in it. These two pilot plants treated the wastewater flowing out from the dissolved air flotation (DAF) unit placed in the first water loop of the paper mill, which is the most contaminated water of the mill. For the lab scale trials the same wastewater was used.

Samples were collected before and after every step of the pilot plants A and B. At pilot-scale the following treatments were compared: anaerobic biological treatment followed by aerobic biological treatment, UF and RO in pilot plant A; and anaerobic biological treatment followed by membrane bioreactor and RO in pilot plant B (Table 2). At lab scale, samples were collected prior and after the treatment by four different AOPs (Fenton reaction, photo-Fenton reaction, photocatalysis by TiO₂ and ozonation). Samples were collected in 2.5 L glass bottles for the analyses of chemical oxygen demand (COD) and selected EDCs. Sample bottles were refrigerated during transportation to the laboratory in order to prevent chemical changes. The samples were taken in the period between February 2009 and July 2009. Analyses were repeated three times for each AOP treatment and each pilot plant in time intervals of few days. A one-way ANOVA (SigmaStat 2.0) was used to determine the significant level of
differences among experimental runs. Post hoc pairwise comparisons were performed using Tukey's test (P<0.05).

**Table 2: Pilot plants used and treatments applied.**

<table>
<thead>
<tr>
<th>pilot plant</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>pilot plant A</td>
<td>anaerobic (An)</td>
</tr>
<tr>
<td></td>
<td>aerobic (Aer)</td>
</tr>
<tr>
<td></td>
<td>ultrafiltration (UF)</td>
</tr>
<tr>
<td></td>
<td>reverse osmosis (RO)</td>
</tr>
<tr>
<td>pilot plant B</td>
<td>anaerobic (An)</td>
</tr>
<tr>
<td></td>
<td>membrane bioreactor (MBR)</td>
</tr>
<tr>
<td></td>
<td>reverse osmosis (RO)</td>
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</table>

2.5.3 Analysis of chemical oxygen demand

COD was measured, according to the Standard Methods for the Examination of Water and Wastewater (APHA 2005), using the colorimetric method at 600 nm with an Aquamate-spectrophotometer (AQA 091801).

3.5.4 Analysis of selected endocrine disrupting compounds

The chemical analysis of selected EDCs was performed using GC-MS as described in Materials and methods (see Section 3.2.2).
3.5.5 Paper mill wastewater treatments from pilot plants

3.5.5.1 Pilot plant A

Pilot plant A consisted of a biological double-step process (anaerobic + aerobic) followed by UF and RO filtration as the final step (Figure 2). The effluent of the anaerobic reactor was discharged into an activated sludge reactor, divided in three successive cascade basins, through which the water flows in series. An aeration system continuously supplied oxygen to the wastewater at a rate of ≥2 mg/L of O₂. The concentration of solids in the mixed liquor (MLTSS) was kept at 2-3 g/L. A secondary sedimentation served for the separation of the activated sludge from the wastewater coming out from the biological stage. The clarified water entered a 0.04 µm UF membrane system. UF membranes were made of hollow-fibres of polyethersulfone, and worked pressurised in a dead-end mode. The RO unit was formed from membrane modules of spiral wounded polymer with a pore size of 0.1 nm operated in cross-flow conditions.

![Diagram of Pilot plant A](image)

**Figure 2:** Scheme of pilot plant A. DAFs: dissolved air flotation units, UF: ultrafiltration, RO: reverse osmosis.
3.5.5.2 Pilot plant B

Pilot plant B consisted of an anaerobic reactor followed by a MBR and a RO filtration as a final step (Figure 3). The effluent from the anaerobic reactor was discharged into the MBR of polyethersulfone hollow-fibre membranes of 50 nm nominal pore size. The water from the MBR entered the RO section of the same structure and operation as in pilot plant A.

Figure 3: Scheme of pilot plant B. DAFs: dissolved air flotation units, MBR: membrane bioreactor, RO: reverse osmosis.
3.5.6 Paper mill wastewater treatments with advanced oxidation processes

The same wastewater inflowing the pilot plants were collected as described above and treated by conventional Fenton method, photo-Fenton process, photocatalysis with TiO$_2$ and ozonation.

3.5.6.1 Conventional Fenton reaction

The experiments were performed in a 3 L reactor filled with 2 L of wastewater. The water was mixed throughout the experiments with a magnetic device. The temperature was adjusted to 25 ºC avoiding an unnecessary consumption of heating energy. Several authors have studied the effect of temperature on COD removal reporting little influence on COD removal at temperatures varying from 25 ºC to 45 ºC (Kang and Hwang 2000; Rivas et al. 2003; Hermosilla et al. 2009a; Zhang et al. 2005). pH was continuously adjusted to pH 3 (±0.1) using 1 mol/L sodium hydroxide (NaOH) or 1 mol/L sulphuric acid. pH 3 has been reported by different authors (Amat et al. 2005; Hermosilla et al. 2009a, 2009b; Kuo 1992; Zhang et al. 2005) as optimal for Fenton reaction. At this pH value the stability of hydrogen peroxide (H$_2$O$_2$) and the solubility of iron are optimal. After temperature and initial pH adjustment, ferrous sulphate (FeSO$_4$) was added to reach the target ferrous ion (Fe$^{2+}$) concentration, corresponding to [H$_2$O$_2$]:[Fe$^{2+}$] ratios of 1.0, 1.5 and 2.0. A ratio of 1.5 was previously determined as optimal for the development of the Fenton reaction in different wastewaters (Zhang et al. 2005, Hermosilla et al. 2009a). Moreover, [H$_2$O$_2$]:[Fe$^{2+}$] ratios of 1.0 and 2.0 were tested. H$_2$O$_2$ was added in batch mode until the target concentration was reached, which was calculated based upon the stoichiometric ratio according to COD (Kim et al. 1997) of wastewater: 1 g COD = 1 g O$_2$ = 0.03125 mol O$_2$ = 0.0625 mol H$_2$O$_2$ = 2.125 g H$_2$O$_2$. Therefore COD (mg/L) x 2.125 = mg/L of H$_2$O$_2$. Just before the addition of H$_2$O$_2$, Fe$^{2+}$, total iron concentration, COD and selected EDCs were measured. This was set as a reaction time of zero. Aliquots of the treated water were taken at pre-defined time intervals (0, 5, 10, 20 and 30 min) with a syringe. Each aliquot was split into two portions. The first portion was filtered through a 0.45 µm Albet nylon syringe filter to collect the filtrate for determination of the residual H$_2$O$_2$, Fe$^{2+}$ and total iron content. Hydrogen peroxide content was analyzed using the titanium sulphate spectrophotometric method (Pobiner 1961). Soluble ferrous ion
concentration ([Fe$^{2+}$]) was determined using the 1,10-phenanthroline colorimetric method (APHA 2005). To avoid the interference of ferric ion, ammonium fluoride was chosen as the masking agent (Vogel et al. 2000). According to the standard methods (APHA 2005), total soluble iron concentration was determined reducing ferric ion to ferrous ion by hydroxylamine under acidified conditions, and then, its concentration was measured as described above. Ferric ion concentration was estimated by the subtraction of ferrous ion concentration from the total iron concentration. The other non-filtered portion was adjusted to pH=8 with 10 M NaOH, stirred in a beaker for 20 min with a magnetic stirring bar and centrifuged for 10 min at 2000 rpm for the analysis of turbidity, COD, selected EDCs and H$_2$O$_2$ in the supernatant. Measured value of H$_2$O$_2$ was used to correct COD determinations according to Hermosilla et al. (2009a).

3.5.6.2 Photo-Fenton reaction

The experimental protocol for photo-Fenton reaction was the same as the one described above for conventional Fenton reaction with the additional use of a 450 W high-pressure mercury immersion UV lamp (7825-34, ACE-glass, Vineland, USA) located vertically in the centre of the reactor. The UV lamp was enclosed inside a quartz glass vessel through which the water was circulated in order to reduce the excessive heat generated during the UV irradiation. The entire assembly was in a safety cabinet (7836-20, Ace-glass, Vineland, USA). Light intensity was recorded using a radiometer (UV-Elektronik, UV-VIS Radiometer RM-21, Ettlingen, Germany), resulting in 186 mW/cm$^2$ between 315 to 400 nm, at the mid-height of the UV lamp, and 1.5 cm from the light source, which was the distance between sample and irradiation source. Figure 4 shows necessary equipment for UV irradiation. After temperature and initial pH adjustment, FeSO$_4$ was added to reach the target Fe$^{2+}$ concentrations, corresponding to an [H$_2$O$_2$]:[Fe$^{2+}$] ratio of 32 that has been shown as optimal for the treatment of different wastewaters (Hermosilla et al. 2009a). The irradiation of UV light allows the reduction of ferric to ferrous iron in photo-assisted Fenton processes (Kavitha and Palanivelu 2004; Hermosilla et al. 2009b). Moreover, the increase of turbidity reduces the efficiency of the treatment (Kim et al. 1997). Therefore, a lower [H$_2$O$_2$]:[Fe$^{2+}$] ratio is needed for the optimization of the photo-Fenton process. H$_2$O$_2$ was added in batch mode until the target concentration was reached, which was calculated according to Kim et al. (1997), as described
above for the conventional Fenton reaction. The influence of the double amount of H₂O₂ (COD (mg/L) x 4.250 = mg/L of H₂O₂) was tested for the photo-Fenton process. The UV lamp was switched on simultaneously when the H₂O₂ was added. Just before the addition of H₂O₂; Fe²⁺, total iron concentration, COD and selected EDCs were measured. This was set as a reaction time zero. Aliquots of the treated water were taken at pre-defined time intervals (0, 20, 40, 60, 80, 100 and 120 min) with a syringe. Each aliquot was split into two portions. The first portion was filtered through a 0.45 μm Albet nylon syringe filter to collect the filtrate for the determination of the residual H₂O₂, Fe²⁺ and total iron content. The other non-filtered portion was adjusted to pH=8 with 10 M NaOH, stirred in a beaker for 20 min with a magnetic stirring bar and centrifuged for 10 min at 2000 rpm for the analysis of turbidity, COD, selected EDCs and H₂O₂ in the supernatant.

3.5.6.3 Photocatalysis with TiO₂ reagent

Samples were filtered through 1 μm glass microfiber filters (Filter-lab MFV) prior to the treatment with TiO₂. Photocatalysis with TiO₂ is less oxidative process than conventional Fenton and photo-Fenton processes; therefore, it is necessary to improve the efficiency of the transmission of the UV light. In a full scale treatment, suspended solids can be removed by normal filtration systems as sand filters; 5 and 10 g/L of TiO₂ were added to the samples, which were UV irradiated with a 450 W high-pressure mercury immersion lamp (7825-34, ACE-glass, Vineland, USA) for 180 min. The entire assembly was in a safety cabinet (7836-20, Ace-glass, Vineland, USA). Figure 4 shows necessary equipment for UV irradiation. The efficiency of the photocatalysis process and the required reaction time for its maximization are influenced by the dosage of TiO₂ (Chang et al. 2004). The degradation of organic compounds is improved until an excessive catalyst dosage causes a shadow effect interfering with the transmission of UV light; therefore, electron–hole pair generation cannot effectively occur. It has been reported that 10 g/L TiO₂ is the optimal level for the treatment of lignin powder (Chang et al. 2004). Aliquots of the treated water were filtered through 0.45 μm filters every 30 min from the beginning of the trial for measuring turbidity, COD and selected EDCs.
Figure 4: Scheme of lab equipment for UV irradiation.
3.5.6.4 Ozonation

Ozonation experiments were conducted in a glass jacketed cylindrical bubble reactor (high=1 m, diameter=5 cm) with a continuous feed of gas (4.0 L/min) with an ozone concentration in the gas of 13 g/Nm$^3$, produced from ordinary grade air passed through polycarbonate filters and subsequently enriched with oxygen. The system consisted of an ozone generator (Model 6020, Rilize, Gijón, Spain), a flow controller Bronkhorst® (Model F-201AV, Ruurlo, The Netherlands) and an ozone on-line analyser (Model 964C, BMT Messtechnic GMBH, Berlin, Germany). The unconsumed ozone was sent to a catalytic ozone constructor. A peristaltic pump (Masterflex® Console Drive, Cole-Parmer Instrument Company, Illinois, USA) was used to recirculate the solution under treatment (1 L) through reactor and probes for the measurement of pH, redox potential and dissolved oxygen (ProODO, YSI Inc., Ohio, USA). The temperature was kept at 25 °C using a thermostatic bath (Model FL300, Julabo Labortechnic GmbH, Seelbach, Germany), which was aided by the reactor glass jacket itself. Additional experiments were performed with the addition of 50 mM of H$_2$O$_2$ into the ozonation reactor.

![Scheme of lab equipment for ozone treatment.](image)

**Figure 5**: Scheme of lab equipment for ozone treatment.
3.5.7 Removal efficiency calculations

For the pilot plant trials removal efficiency calculations of the COD and selected EDCs are based on the differences between the concentrations at the inflow and at the outflow of each treatment step of the pilot plants A and B (Figure 2 and 3). For the lab scale AOP trials removal efficiency calculations of the COD and selected EDCs are based on the differences between the initial concentrations and concentrations after pre-defined time intervals.
4. RESULTS AND DISSCUSSION

4.1 Determination of selected endocrine disrupting compounds in two Slovenian paper mill wastewaters

The aim of this part of the research was the optimization of chemical analytical protocol and chemical characterisation for the effluents from paper making facilities with the main intention to determine the levels of DMP, DEP, DBP, BBP, BEHP, BPA and NP in raw and biologically treated paper mill effluents and associated surface waters.

SPE and derivatization step proved to be an efficient method for preparation of water samples for GC-MS analysis. SPE is known as a very sensitive method for trace analyses of organic pollutants in different types of aqueous matrices. It was found out that GC-MS combined with SPE after derivatization with BSTFA agent can be used to determine trace levels of DMP, DEP, DBP, BBP, BEHP, BPA and NP in water samples. Using this approach the GC-MS analysis confirmed all seven EDCs in the effluents from both Slovenian paper mills with different production process. Paper mill A is manufacturing label papers and flexible packaging papers from fresh cellulose fibres and has on site aerobic biological wastewater treatment plant. Paper mill B is manufacturing carton board from 100% recovered fibres and has on site combined aerobic-anaerobic biological wastewater treatment plant.

The concentrations of DMP, DEP, DBP, BBP, BEHP, BPA and NP differed significantly according to the production process. Maximum values of phthalates in paper mill wastewaters before treatment reached 220.41 µg/L (DBP) for paper mill A and 349.61 µg/L (DMP) for paper mill B. Average values of phthalates in paper mill wastewaters before treatment are presented in Table 3. After the biological treatment, phthalates values in both paper mill effluents significantly decreased (Table 3). Maximum values of BPA in paper mill wastewaters before treatment reached 31.7 µg/L for paper mill A and 93.82 µg/L for paper mill B. Average values of BPA in paper mill wastewaters before treatment are presented in Table 3. After the biological treatment, BPA values in both paper mill effluents significantly
decreased (Table 3). Maximum values of NP in raw paper mill effluents reached 10.6 µg/L for paper mill A and 41.15 µg/L for paper mill B. Average values of NP in paper mill wastewaters before treatment are presented in Table 3. After the biological treatment, NP values in both paper mill effluents significantly decreased. Possible reason for reduction of selected phthalates, BPA and NP concentrations after biological wastewater treatment is most probably biodegradation during biological treatment. Average values of DMP, DEP, DBP, BBP, BEHP, BPA and NP in paper mill effluents before and after biological treatment (sampling locations 1A and 1B; and 2A and 3B according Table 1) are presented in Table 3.

Among identified compounds, BEHP and NP are on the list of priority substances according to Directive (2008/105/EC) and bisphenol A is identified as substance subject to review for possible identification as priority substance or priority hazardous substance. According to Directive (2008/105/EC) the annual average limit value for surface waters for BEHP and NP are 1.3 µg/L and 0.3 µg/L; and the maximum allowable concentrations for NP detected in surface waters is 2.0 µg/L, respectively. For other four investigated compounds there are no regulations for the environment yet, although DMP, DEP, DBP and BBP are detrimental already if present in low concentration levels and they should be seriously considered and their impact studied.

Our results showed, that biological treated paper mill effluents from paper mill A met the above mentioned standards for BEHP in period from August 2008 till November 2008 and September 2009, but not in the period from March 2009 till May 2009. Average values for BEHP in recovered paper mill effluents (sampling location 2 in the case of paper mill A) were 0.21 ± 0.04 µg/L (August-November 2008), 1.23 ± 0.27 µg/L (September 2009) and 1.62 ± 0.6 µg/L (March-May 2009). The biological treated paper mill effluents from paper mill B met the above mentioned standards for BEHP in period from August 2008 till November 2008, but not in the period from March 2009 till May 2009, and January 2010. Average values for BEHP in recovered paper mill effluents (sampling location 3B) were 0.38 ± 0.05 µg/L (August-November 2008), 1.35 ± 0.45 µg/L (March-May 2009) and 1.73 ± 1.08 µg/L (January 2010). Directive (2008/105/EC) does not specify maximum allowable concentrations for BEHP.
Our results showed, that biological treated paper mill effluents from paper mill A did not meet the above mentioned standards for NP in period from August 2008 till November 2008, March 2009 till May 2009 and September 2009. At these periods, the average values for NP in biologically treated paper mill effluents (sampling location 2A in the case of paper mill A) were 0.34 ± 0.05 µg/L for paper mill A (August-November 2008), 0.31 ± 0.09 µg/L (March-May 2009) and 0.32 ± 0.07 µg/L (September 2009). The biological treated paper mill effluents from paper mill B met the above mentioned standards for NP in period from March 2009 till May 2009, but not in the period from August-November 2008 and January 2010. From obtained data it can be concluded that paper industry use NP as cleaning agents and/or additives in dispersants, antifoamers and deinkers. Average values for NP in recovered paper mill effluents (sampling location 3B) were 0.29 ± 0.09 µg/L (March-May 2009), 0.60 ± 0.12 µg/L (August-November 2008) and 0.52 ± 0.21 µg/L (January 2010). Concentrations of NP found in our study, agrees with those from Rigol et al. (2004). None of the samples exceeded the maximum allowable concentrations for NP (2.0 µg/L) according to Directive (2008/105/EC). According to the GC-MS measurements, there was not significant difference between the paper mill wastewaters. However, concentrations of investigated substances in paper mill wastewaters vary greatly. In 2008, concentrations of substances in both paper mill wastewaters were much lower. The reason for these observations may be due to changes in the manufacturing process or substitution of raw materials and additives.
Table 3: Average concentrations with ± SD of selected EDCs [dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), bis(2-ethylhexyl) phthalate (BEHP), bisphenol A (BPA) and nonylphenol (NP)] in raw (sample location 1A and 1B) and biologically treated paper mill wastewaters (sample location 2A and 3B) according to the paper mills.

Exceeded legislation values (after treatment; sample location 2A and 3B) are marked in bold (Directive (2000/60/EC and 2008/105/EC)).

<table>
<thead>
<tr>
<th>Compound</th>
<th>paper mill A</th>
<th>paper mill B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>August – November 2008</td>
<td>March – May 2009</td>
</tr>
<tr>
<td></td>
<td>before treatment (sample location 1A)</td>
<td>after treatment (sample location 2A)</td>
</tr>
<tr>
<td></td>
<td>Average concentrations ±SD (µg/L)</td>
<td>Average concentrations ±SD (µg/L)</td>
</tr>
<tr>
<td>DMP</td>
<td>6.12±0.08</td>
<td>0.75±0.02</td>
</tr>
<tr>
<td>DEP</td>
<td>0.15±0.03</td>
<td>0.02±0.03</td>
</tr>
<tr>
<td>DBP</td>
<td>0.19±0.04</td>
<td>0.04±0.01</td>
</tr>
<tr>
<td>BBP</td>
<td>1.25±0.18</td>
<td>0.16±0.05</td>
</tr>
<tr>
<td>BEHP</td>
<td>1.32±0.07</td>
<td>0.21±0.04</td>
</tr>
<tr>
<td>BPA</td>
<td>3.75±0.91</td>
<td>0.99±0.15</td>
</tr>
<tr>
<td>NP</td>
<td>1.32±0.18</td>
<td><strong>0.34±0.05</strong></td>
</tr>
<tr>
<td>Compound</td>
<td>September 2009</td>
<td>January 2010</td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
<td>--------------</td>
</tr>
<tr>
<td></td>
<td>before treatment</td>
<td>after treatment</td>
</tr>
<tr>
<td>(sample location 1A)</td>
<td>(sample location 2A)</td>
<td>(sample location 1B)</td>
</tr>
<tr>
<td>DMP</td>
<td>3.99±2.47</td>
<td>0.69±0.38</td>
</tr>
<tr>
<td>DEP</td>
<td>6.77±2.36</td>
<td>1.14±0.4</td>
</tr>
<tr>
<td>DBP</td>
<td>10.78±4.58</td>
<td>1.22±0.41</td>
</tr>
<tr>
<td>BBP</td>
<td>9.88±1.99</td>
<td>1.09±0.21</td>
</tr>
<tr>
<td>BEHP</td>
<td>13.42±4.25</td>
<td>1.23±0.27</td>
</tr>
<tr>
<td>BPA</td>
<td>11.38±3.37</td>
<td>1.91±0.39</td>
</tr>
<tr>
<td>NP</td>
<td>6.43±0.95</td>
<td>0.32±0.07</td>
</tr>
</tbody>
</table>

4.2 Determination of mutagenic/genotoxic activity in two Slovenian paper mill wastewaters

4.2.1 Determination of selected endocrine disrupting compounds

The chemical analysis done in September 2009 (paper mill A) and January 2010 (paper mill B) revealed that all seven EDCs (DMP, DEP, DBP, BBP, BEHP, BPA and NP) were confirmed in raw and biologically treated effluents from paper mill A and paper mill B. In the associated surface waters DMP, DEP, BBP and BPA were not detected. However, in the case of paper mill A, BEHP and NP were found in the recipient downstream surface waters (rivers) in the concentrations 0.21 ± 0.03 µg/L (Wednesday) and 0.1 ± 0.01 µg/L (Friday) and 0.19 ± 0.02 µg/L (Saturday). In the case of paper mill B, DBP was found in the recipient downstream surface waters (rivers) in the concentrations 0.1 ± 0.02 µg/L (Saturday), 0.1 ± 0.01 µg/L (Thursday) and 0.33 ± 0.01 µg/L (Wednesday). The average with standard deviations for all seven determined EDCs in both paper mills are presented in Figure 6 and 7.
Dimethyl phthalate

Diethyl phthalate

Dibutyl phthalate

Benzyl butyl phthalate
Figure 6: Average (± SD) for dimethyl phthalate, diethyl phthalate, dibutyl phthalate, benzyl butyl phthalate, bis(2-ethylhexyl) phthalate, bisphenol A and nonylphenol in paper mill A. n.d. (not detected). The SD values are to small to be visible on the graphs.

1A – before aerobic treatment; 2A – after aerobic treatment (before discharge into the surface water).

Exceeded legislation values are marked with bold ↓ (Directive (2000/60/EC and 2008/105/EC)).
Dimethyl phthalate

Diethyl phthalate

Dibutyl phthalate

Benzyl butyl phthalate

sampling day

concentrations (µg/L)

A

B

C

D

1B
2B
3B

1B
2B
3B

1B
2B
3B

1B
2B
3B

1B
2B
3B
Figure 7: Average (± SD) for dimethyl phthalate, diethyl phthalate, dibutyl phthalate, benzyl butyl phthalate, bis(2-ethylhexyl) phthalate, bisphenol A and nonylphenol in paper mill B. The SD values are too small to be visible on the graphs.
1B – after anaerobic treatment; 2B – before aerobic treatment (after aerobic treatment); 3B – after aerobic treatment (before discharge into the surface water).
Exceeded legislation values are marked in bold ↓ (Directive (2000/60/EC and 2008/105/EC).

Our results showed (Figure 6 and 7), that raw paper mill effluents from both paper mills and effluent after anaerobic WWTP in paper mill B exceeded the limit values for BEHP and NP, however effluent after biological WWTP in paper mill A exceeded the limit value for BEHP in three measurements and for NP in four measurements. Effluent after aerobic BWTP in paper mill B exceeded limit value for BEHP in three measurements and for NP in six measurements. BPA suspected to migrate from additives, showed 80% degradation after aerobic wastewater treatment (paper mill A) and 70% degradation after anaerobic wastewater treatment (paper
mill B) followed by addition 70% degradation after aerobic wastewater treatment (paper mill B). Concentrations of BPA found in our study, agrees with those from Fürhacker et al. (2000) and Terasaki et al. (2007). Surfactants, such as alkylphenol ethoxylates are present in paper mill wastewaters because of their use as cleaning agents and/or additives in dispersants, antifoamers and deinkers. The alkylphenol ethoxylates degrade to NP, which is considered as persistent environmental pollutants (Latorre et al. 2005). Concentrations of NP found in our study, agrees with those from Rigol et al. (2004). According to a study from 2008 (the same paper mills, sampling in the months of August to November), (Balabanič and Krivograd Klemenčič 2011) was found that concentrations of investigated substances in paper mill wastewaters vary greatly. In 2008, concentrations of substances in both paper mill wastewaters were much lower. The reason for these observations may be due to changes in the manufacturing process or substitution of raw materials and additives. Production program in paper industry may be frequently changing (up to 3 times per day).

4.2.2 Mutagenic/genotoxic activity

4.2.2.1 Bacterial assays

4.2.2.1.1 SOS/umuC ASSAY

The results of the SOS/umuC assay for 7 day- sampling period (September 2009 for paper mill A effluents and January 2010 for paper mill B effluents) are shown in Tables 4 and 5.
Table 4: The effect of paper mill A wastewater on the induction of SOS response in Salmonella typhimurium TA1535/pSK1002.

<table>
<thead>
<tr>
<th>sampling day and place*</th>
<th>without S9</th>
<th>with S9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G^a ± SD</td>
<td>IR^b ± SD</td>
</tr>
<tr>
<td>control</td>
<td>1.0±0.04</td>
<td>1.0±0.08</td>
</tr>
<tr>
<td>Monday 1A</td>
<td>1.23±0.06</td>
<td>0.9±0.09</td>
</tr>
<tr>
<td>Monday 2A</td>
<td>1.09±0.11</td>
<td>0.87±0.17</td>
</tr>
<tr>
<td>Monday 3A</td>
<td>1.05±0.09</td>
<td>0.91±0.03</td>
</tr>
<tr>
<td>Monday 4A</td>
<td>1.06±0.11</td>
<td>0.95±0.01</td>
</tr>
<tr>
<td>Tuesday 1A</td>
<td>1.07±0.01</td>
<td>0.99±0.08</td>
</tr>
<tr>
<td>Tuesday 2A</td>
<td>0.98±0.11</td>
<td>0.94±0.15</td>
</tr>
<tr>
<td>Tuesday 3A</td>
<td>1.02±0.07</td>
<td>0.92±0.19</td>
</tr>
<tr>
<td>Tuesday 4A</td>
<td>1.03±0.05</td>
<td>0.91±0.11</td>
</tr>
<tr>
<td>Wednesday 1A</td>
<td>1.14±0.06</td>
<td>0.88±0.17</td>
</tr>
<tr>
<td>Wednesday 2A</td>
<td>1.12±0.07</td>
<td>0.89±0.14</td>
</tr>
<tr>
<td>Wednesday 3A</td>
<td>1.03±0.05</td>
<td>1.02±0.06</td>
</tr>
<tr>
<td>Wednesday 4A</td>
<td>1.05±0.05</td>
<td>1.01±0.06</td>
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<tr>
<td>Thursday 1A</td>
<td>1.06±0.03</td>
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<tr>
<td>Thursday 2A</td>
<td>1.01±0.02</td>
<td>1.1±0.01</td>
</tr>
<tr>
<td>Thursday 3A</td>
<td>0.96±0.06</td>
<td>0.98±0.21</td>
</tr>
<tr>
<td>Thursday 4A</td>
<td>0.98±0.06</td>
<td>0.95±0.21</td>
</tr>
<tr>
<td>Friday 1A</td>
<td>1.05±0.05</td>
<td>0.97±0.23</td>
</tr>
<tr>
<td>Friday 2A</td>
<td>1.04±0.08</td>
<td>1.02±0.12</td>
</tr>
<tr>
<td>Friday 3A</td>
<td>1.01±0.04</td>
<td>1.06±0.11</td>
</tr>
<tr>
<td>Friday 4A</td>
<td>0.99±0.1</td>
<td>1.14±0.06</td>
</tr>
<tr>
<td>Saturday 1A</td>
<td>1.05±0.04</td>
<td>1.27±0.01</td>
</tr>
<tr>
<td>Saturday 2A</td>
<td>1.03±0.08</td>
<td>1.22±0.17</td>
</tr>
<tr>
<td>Saturday 3A</td>
<td>0.99±0.08</td>
<td>1.22±0.11</td>
</tr>
<tr>
<td>Saturday 4A</td>
<td>0.99±0.06</td>
<td>1.11±0.37</td>
</tr>
<tr>
<td>sampling day and place*</td>
<td>without S9</td>
<td>with S9</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td>$G^a \pm SD$</td>
<td>$IR^b \pm SD$</td>
</tr>
<tr>
<td>control</td>
<td>1.0±0.04</td>
<td>1.0±0.08</td>
</tr>
<tr>
<td>Sunday 1A</td>
<td>1.03±0.05</td>
<td>1.25±0.04</td>
</tr>
<tr>
<td>Sunday 2A</td>
<td>1.06±0.05</td>
<td>1.22±0.18</td>
</tr>
<tr>
<td>Sunday 3A</td>
<td>1.04±0.08</td>
<td>1.3±0.13</td>
</tr>
<tr>
<td>Sunday 4A</td>
<td>1.04±0.09</td>
<td>1.27±0.18</td>
</tr>
</tbody>
</table>

* Bacterial growth rate $G$: the ratio of the bacterial growth of the water sample treated bacteria to that of the distilled water control; $G < 0.75$ is considered as toxic for bacteria.

* Induction ratio $IR$: the ratio of β-galactosidase activity of the water sample treated bacteria to that of the distilled water control; $IR > 1.5$ is considered as genotoxic response.

*a* - before aerobic treatment, 2 - after aerobic treatment/before released into surface water, 3 - upstream, 4 - downstream

Table 5: The effect of paper mill B wastewater on the induction of SOS response in Salmonella typhimurium TA1535/pSK1002.

<table>
<thead>
<tr>
<th>sampling day and place*</th>
<th>without S9</th>
<th>with S9</th>
</tr>
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<td>sampling day and place*</td>
<td>without S9</td>
<td>with S9</td>
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<tr>
<td>Sunday 5B</td>
<td>1.05±0.08</td>
<td>1.17±0.12</td>
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</tbody>
</table>

* Bacterial growth rate $G$: the ratio of the bacterial growth of the water sample treated bacteria to that of the distilled water control; $G < 0.75$ is considered as toxic for bacteria.
Induction ratio $IR$: the ratio of $\beta$-galactosidase activity of the water sample treated bacteria to that of the distilled water control; $IR > 1.5$ is considered as genotoxic response.

*1 - before anaerobic treatment, 2 - after anaerobic treatment/before aerobic treatment, 3 - after aerobic treatment/before released into surface water, 4 - upstream, 5 - downstream

Samples promoting a decrease in the bacterial growth rate ($G$) during incubation of more than 25% compared to controls were defined as cytotoxic. The viability of $S. typhimurium$ TA1535/pSK1002 strain cells exposed to untreated and biologically treated wastewaters from two Slovenian paper mills and associated surface waters was not significantly affected. Furthermore, the untreated and biologically treated wastewaters from two Slovenian paper mills and associated surface waters did not increase $\beta$-galactosidase activity in the SOS/umuC assay. The addition of S9 enzyme fraction did not have any influence on genotoxic activity of untreated and biologically treated wastewaters from both Slovenian paper mills and associated surface waters.

4.2.2.1.2 AMES MPF™ 98/100 Aqua ASSAY

Un-concentrated untreated and biologically treated samples of wastewater from paper mill A and associated surface waters were not significantly mutagenic in the test system Ames MPF™ 98/100 Aqua assay (data not shown). However, more than half of the untreated wastewater samples from paper mill B were mutagenic in the Ames MPF™ 98/100 Aqua assay with $S. typhimurium$ strains TA100 with metabolic activation S9. This mutagenic effect was not observed after anaerobic and aerobic treatment, indicating that biodegradation or adsorptive elimination and respective sedimentation of mutagenic compounds during biological treatment of influent. Samples which were taken on Sunday were special because they were mutagenic with both S9 metabolic activation and without it, and were mutagenic even after biological treatment. Similar results were also obtained in wastewaters from Baikalsk Pulp and Paper mill in Russia (Lindström-Seppä et al. 1998). Figure 8 shows the results of diluted samples which were positive in Ames MPF™ 98/100 Aqua assay. Four wastewater samples from paper mill B (Wednesday 1B, Friday 1B, Saturday 1B and Sunday 1B) from the same location (before anaerobic treatment) contained promutagens that needed metabolic activation to become mutagens (Figure 8C, 8D, 8E and 8F). Liver enzymes that
normally detoxify harmful metabolic intermediates are often responsible for the activation of promutagens into mutagens. The positive samples were repeated at serial dilutions (1:1, 1:2, 1:4) and the results showed dose dependent mutagenic activity of tested samples (Figure 8).

**Figure 8:** The mutagenic effect of paper mill B wastewater in Ames MPF™ 98/100 Aqua assay with Salmonella typhimurium TA100 strain.
Bacteria (TA100) were exposed to different dilutions of wastewater samples (1:4, 1:2, 1:1) in the absence (-S9) and presence (+S9) of metabolic activation. Location 1 is before anaerobic treatment and location 3 is after aerobic treatment/before released into surface water; BL - base line (---------); * - Fold inc. over BL; the Xenometrix program was used for calculation the mutagenic activity of un-concentrated samples (Xenometrix 2010).

A) Bacteria exposed to sample Sunday 1B in the absence of metabolic activation S9; B) Bacteria exposed to sample Sunday 3B in the absence of metabolic activation S9; C) Bacteria exposed to sample Wednesday 1B in the presence of metabolic activation S9; D) Bacteria exposed to sample Friday 1B in the presence of metabolic activation S9; E) Bacteria exposed to sample Saturday 1B in the presence of metabolic activation S9; F) Bacteria exposed to sample Sunday 1B in the presence of metabolic activation S9.

Ames MPF™ 98/100 Aqua assay proved to be more sensitive for mutagenic compounds in wastewater samples than the SOS/umuC assay. The reason could be that by Ames MPF™ 98/100 Aqua assay the frameshifts or base-pair substitution mutations are detected, while by SOS/umuC assay the activation of the SOS response in the bacteria and recording the β-galactosidase activity from an integrated reporter system are measured. We found previous reports that effluents from kraft mills and effluents after the chlorination step cause mainly base-pair substitution mutations in S. typhimurium strains TA100 without metabolic activation (Rao et al., 1995, Lindström-Seppä et al. 1998).
**4.2.2.2 Eucaryotic assay**

**4.2.2.2.1 CYTOTOXICITY ASSAY (MTT ASSAY)**

First the cytotoxicity was determined in order to select non-toxic concentrations and to avoid false positive/negative responses in genotoxicity assays due to cytotoxicity and not genotoxicity. The viability of HepG2 cells exposed to 30 vol.% of paper mill wastewaters for 24 h was not significantly affected (data not shown). Therefore, these concentrations were used in further experiments.

**4.2.2.2.2 SINGLE CELL GEL ELECTROPHORESIS ASSAY (COMET ASSAY)**

The genotoxic activity of un-concentrated untreated and treated samples of wastewater from paper mill A and associated surface waters, was determined using comet assay. The results showed that after 24h of exposure none of the water samples (30 vol.%) induced increased DNA damage in HepG2 cells (data not shown). On contrary, more than two thirds (Monday, Tuesday, Wednesday, Friday and Sunday) of untreated wastewater samples from paper mill B (sample location 1B) increased % of tail DNA in human derived cells (Figure 9). The positive results were confirmed by testing the samples in serial dilution (0, 10, 20 and 30 vol.% of water sample). These results suggest that genotoxic compounds are present in the effluents from paper mill B, which are subsequently subjected to wastewater treatment processes.
Figure 9: The level of DNA damage induced by untreated paper mill effluents (sample location 1B). The HepG2 cells were exposed to the 0, 10, 20 and 30 vol.% of water samples for 24 h. DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25\textsuperscript{th} and 75\textsuperscript{th} percentiles, the mean value is a solid line through the box.
* Denotes a significant difference between the control (30 vol.% of distilled water) and the water sample treated cells (Kruskal-Wallis test, p < 0.05).

After anaerobic treatment (sample location 2B) all samples showed genotoxic activity (Figure 10). From these data, it can be concluded that in the phase of anaerobic treatment process water is not efficiently cleaned and genotoxic compounds are still present. Furthermore, probably additional genotoxic compounds are formed and are released to the next treatment step. This conclusion is derived from the observation that before biological treatment the genotoxic activity was lower.
Figure 10: The level of DNA damage induced by anaerobically treated paper mill effluents (sample location 2B). The HepG2 cells were exposed to the 0, 10, 20 and 30 vol.% of water samples for 24 h. DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25th and 75th percentiles, the mean value is a solid line through the box.

* Denotes a significant difference between the control (30 vol.% of distilled water) and the water sample treated cells (Kruskal-Wallis test, p < 0.05).

Next step of paper mill B wastewater treatment process is aerobic treatment (sample location 3B). More than two thirds (Monday, Tuesday, Wednesday, Thursday and Friday) of the samples taken after aerobic treatment (sample location 3B) were positive in comet assay
(Figure 11). Genotoxicity of wastewaters after aerobic treatment indicates poor treatment efficiency of paper mill wastewaters treatment plant. Our previous study showed that combination of anaerobic and aerobic wastewater treatments efficiently reduced the COD and selected EDCs from wastewaters after biological treatments in paper mill B. The reason for relatively high genotoxic activity of wastewaters after aerobic treatment could be in synergistic effects between components; therefore new methods for efficient reduction of genotoxic compounds are necessary.
Figure 11: The level of DNA damage induced by aerobically treated paper mill effluents (sample location 3B). The HepG2 cells were exposed to the 0, 10, 20 and 30 vol.% of water samples for 24 h. DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25th and 75th percentiles, the mean value is a solid line through the box.
* Denotes a significant difference between the control (30 vol.% of distilled water) and the water sample treated cells (Kruskal-Wallis test, p < 0.05).

The surface water samples that were taken upstream of the discharge from paper mill B (sample location 4B) were negative in comet assay, with an exception of the sample taken on Wednesday (Figure 12). On the contrary, more than two thirds (Monday, Wednesday, Thursday, Friday and Saturday) of the surface water samples downstream of the discharge from paper mill B (sample location 5B) increased the formation of strand breaks in HepG2 cells (Figure 13). The positive results were confirmed by testing the water samples in serial dilutions (0, 10, 20 and 30 vol.% of water sample). From these data, it can be concluded that effluent from paper mill B exhibited significant genotoxic effect.

**Figure 12**: The level of DNA damage induced by surface water upstream of the discharge of effluents to the river (sample location 4B). The HepG2 cells were exposed to the 0, 10, 20 and 30 vol.% of water samples for 24 h. DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25\textsuperscript{th} and 75\textsuperscript{th} percentiles, the mean value is a solid line through the box.
* Denotes a significant difference between the control (30 vol.% of distilled water) and the water sample treated cells (Kruskal-Wallis test, \( p < 0.05 \)).

Figure 13: The level of DNA damage induced by surface water downstream of the discharge of effluents to the river (sample location 5B). The HepG2 cells were exposed to the 0, 10, 20 and 30 vol.% of water samples for 24 h. DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of
the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25th and 75th percentiles, the mean value is a solid line through the box.

* Denotes a significant difference between the control (30 vol.% of distilled water) and the water sample treated cells (Kruskal-Wallis test, p < 0.05).

The obtained results indicate that even after the treatment genotoxic substances are introduced into environment, thus potentially presenting the hazard for indigenous organisms.

The most likely reason for the high mutagenic/genotoxic activity of investigated wastewater samples is the complex assortment of chemicals used in the paper production processes, the release of which is not controlled with limited targeted chemical and biological analyses. Recovering fibers and recycling them into new paper, decreases the demand for natural resources, saves energy and water and reduces waste going to landfills. However, recovered fibres may contain residues of fillers, biocides and inks which may contribute to the genotoxic activity of exposed wastewaters. Therefore more attention is needed in wastewater treatment processes to obtain clean wastewater, which is released to the environment.

In the investigated paper mill wastewaters and associated surface waters traces of DMP, DEP, DBP, BBP, BEHP, BPA and NP have been detected. We were interested whether they may contribute to the observed genotoxicity. We tested pure and combinations of detected EDCs for induction of DNA damage in HepG2 cells. We selected four real wastewater samples with known amounts of EDCs and prepared artificial samples with the same composition and concentrations of pure EDCs as corresponding real wastewater samples. We selected samples Tuesday 2A (Figure 14A), that contained (0.62±0.03 µg/L DMP, 1.94±0.05 µg/L DEP, 0.65±0.05 µg/L DBP, 1.05±0.01 µg/L BBP, 1.39±0.09 µg/L BEHP, 1.71±0.11 µg/L BPA and 0.42±0.02 µg/L NP), Thursday 2A (Figure 14B), that contained (1.0±0.01 µg/L DMP, 0.79±0.02 µg/L DEP, 1.06±0.01 µg/L DBP, 1.0±0.03 µg/L BBP, 1.56±0.03 µg/L BEHP, 2.01±0.07 µg/L BPA and 0.31±0.01 µg/L NP), Sunday 1B (Figure 14C), that contained (110.84±0.48 µg/L DMP, 25.9±0.44 µg/L DEP, 21.15±0.23 µg/L DBP, 16.85±0.26 µg/L BBP, 26.83±0.35 µg/L BEHP, 28.64±0.34 µg/L BPA and 29.08±0.23 µg/L NP) and sample
Sunday 3B (Figure 14D), that contained (4.03±0.27 µg/L DMP, 2.05±0.06 µg/L DEP, 3.04±0.16 µg/L DBP, 3.99±0.17 µg/L BBP, 4.04±0.17 µg/L BEHP, 5.04±0.07 µg/L BPA and 0.57±0.16 µg/L NP). The results showed that the real sample Tuesday 2A and corresponding artificially mixed sample did not differ in genotoxic activity, while all other samples did. Artificially prepared sample corresponding to sample Thursday 2A did not differ in genotoxic activity, while artificially prepared sample induced slightly more DNA strand breaks comparison with control. Real samples Sunday 1B and 3B induced much higher DNA damage than artificially mix samples. These results suggest that real samples Sunday 1B and 3B contained apart of detected EDCs other compounds with genotoxic potential or compounds that could influence and increase genotoxic potential of detected EDCs. These results again show the importance of combination of chemical analysis and bioassays for better prediction of the genotoxic potential of complex wastewater samples as synergistic or antagonistic effects between the components may occur.
**Figure 14:** The level of DNA damage induced by untreated and treated paper mill effluents and artificially prepared mixtures of EDCs detected in corresponding water samples. The HepG2 cells were exposed to 30 vol.% of water samples and artificially prepared combination of EDCs with the same composition as real samples for 24 h. DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25th and 75th percentiles, the mean value is a solid line through the box.

* Denotes a significant difference between the control and the water sample treated cells (Kruskal-Wallis test, $p < 0.05$).
Similar approach as in our study was previously used on wastewater treatment plant samples and surface water as well as drinking water samples (Dizer et al. 2002; Monarca et al. 2004; Ferk et al. 2009; Žegura et al. 2009). In these studies bioassays were shown to be a very sensitive tool for determination of genotoxic activity of tested samples and the same was confirmed in our study.

### 4.3 Potential mutagenic/genotoxic activity of selected pure endocrine disrupting compounds

As our paper mill wastewaters and associated surface waters that contained EDCs showed genotoxic activity, we used similar approach for testing genotoxic potential of pure EDCs that were detected in our water samples.

**Table 6: Concentrations and molarity of selected compounds [dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), bis(2-ethylhexyl) phthalate (BEHP), bisphenol A (BPA) and nonylphenol (NP)]**

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DMP (M<sub>w</sub>=194.19 g/mol), DEP (M<sub>w</sub>=222.24 g/mol), DBP (M<sub>w</sub>=278.35 g/mol), BBP (M<sub>w</sub>=312.37 g/mol), BEHP (M<sub>w</sub>=390.57 g/mol), BPA (M<sub>w</sub>=228.29 g/mol) and NP (M<sub>w</sub>=220.36 g/mol)
4.3.1 Bacterial assays

The potential cytotoxic and genotoxic activity of different concentrations (0, 1, 10, 100 and 1000 µg/L) of pure EDCs (DMP, DEP, DBP, BBP, BEHP, BPA and NP) was determined with SOS/umuC assay on *S. typhimurium* TA1535/pSK1002 strain. The results are summarized in Table 7. None of the tested EDCs increased SOS/umuC response and did not affect the viability of bacteria.

The literature data regarding the cytotoxic and genotoxic/mutagenic potential of EDCs on bacteria are very limited. We found only the report that DMP, DEP, DBP, BBP and BEHP were not mutagenic in *Salmonella*/microsomal (Ames) assay with *S. typhimurium* TA98, TA100, TA1535, or TA1537 strains in the presence of liver homogenate preparations (S9) from Aroclor 1254-induced male Sprague-Dawley rats and Syrian hamsters (Zeiger et al. 1982). Similarly, also NP was not mutagenic using *Salmonella* mutation assay, TA98 and TA100 strains without metabolic activation, at the concentrations from 0.937 to 9.37 µg/L (Boyacıoğlu et al. 2007).

The results from our study showed that pure EDCs at concentrations detected in paper mill wastewaters (Figure 6 and 7), did not cause genotoxic response in the SOS/umuC assay in the absence and the presence of S9 metabolic activation (Table 6). However, whole range of compounds from paper mill wastewaters are released into the environment (Figure 6 and 7), therefore synergistic and antagonistic effects between the components in the complex mixture may occur, while in the environment there are many more substances that can in the combination with EDCs cause adverse effects.
**Table 7:** The effect of dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), bis(2-ethylhexyl) phthalate (BEHP), bisphenol A (BPA) and nonylphenol (NP) on the induction of SOS response in Salmonella typhimurium TA1535 pSK1002.

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### Salmonella typhimurium TA1535 pSK1002

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µg/L)</th>
<th>S9 - Gᵃ ± SD</th>
<th>S9 - IRᵇ ± SD</th>
<th>S9 + Gᵃ ± SD</th>
<th>S9 + IRᵇ ± SD</th>
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<td>1.0±0.13</td>
<td>1.0±0.07</td>
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<td>BPA</td>
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<td>0.5±0.02</td>
<td>0.86±0.03</td>
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<td>1.28±0.29</td>
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</tbody>
</table>

ᵃ Bacterial growth rate (G): the ratio of the bacterial growth of the selected EDCs treated bacteria to that of the distilled water control; G < 0.75 is considered as toxic for bacteria.

ᵇ Induction ratio (IR): the ratio of β-galactosidase activity of the selected EDCs treated bacteria to that of the distilled water control; IR > 2 is considered as genotoxic response.

#### 4.3.2 Human hepatoma HepG2 cells

To further evaluate the genotoxic potential of pure EDCs, cytotoxicity and genotoxicity were tested on human hepatoma cell line HepG2 using MTT and comet assay, respectively.

#### 4.3.2.1 Cytotoxicity assay (MTT assay)

Cytotoxicity was first determined in order to select suitable concentrations and to avoid false positive/negative responses in genotoxicity assays due to cytotoxicity and not genotoxicity. The viability of HepG2 cells exposed to 0, 1, 10, 100 and 1000 µg/L of pure EDCs for 24 h.
was not significantly affected (data not shown). Therefore, these concentrations were used in further experiments.

4.3.2.2 Single cell gel electrophoresis assay (comet assay)

The potential genotoxic activity was determined after 4 and 24 h of exposure to different concentrations (0, 1, 10, 100 and 1000 µg/L) of pure EDCs (DMP, DEP, DBP, BBP, BEHP, BPA and NP). DNA damage in HepG2 cells was assessed by measuring the % of tail DNA.

In HepG2 cells DMP, DEP, DBP, BBP, BEHP, BPA and NP (Figure 15) increased % of tail DNA at concentrations from 1-1000 µg/L. DNA damage can be observed already after 4 h treatment and DNA damage also remaining after 24 h treatment.
Figure 15: The level of DNA damage induced by selected EDCs. The HepG2 cells were exposed to the different concentrations (0, 1, 10, 100 and 1000 µg/L) of selected EDCs for 4 h (A, C, E, G, I, K and M) and 24 h (B, D, F, H, J, L and N). DNA damage was assessed with comet assay and is expressed as percent of tail DNA. Fifty cells were analysed per experimental point in each of the three independent experiments. Data are presented as quantile box plots with 95% confidence in intervals. The edges of the box represent the 25th and 75th percentiles, the mean value is a solid line through the box.

* Denotes a significant difference between the control and the water sample treated cells (Kruskal-Wallis test, p < 0.05).

The results of chemical analysis showed that concentrations of pure EDCs in the untreated paper mill wastewaters were in the range from 0 to 20.79±0.12 µg/L (in the case of paper mill A; Figure 6) and from 7.9±0.07 µg/L to 110.84±0.48 µg/L (in the case of paper mill B; Figure
7). DMP, DEP, DBP, BBP, BEHP and BPA at concentrations within these ranges induced significant increase in the % of tail DNA after 4 h of exposure, while the relevant concentrations of DMP, DBP, BPA and NP induced elevated DNA damage also after 24 h of exposure (Figure 15). On the contrary, the detected concentrations of EDCs in the treated paper mill wastewaters ranged from 0 to 2.53±0.04 µg/L (in the case of paper mill A; Figure 6) and from 0.23±0.06 µg/L to 4.03±0.27 µg/L (in the case of paper mill B; Figure 7). The results showed that pure DBP, BBP and BEHP were present in treated paper mill wastewaters at concentrations that induced significant DNA damage in HepG2 cells after short exposure time (4 h), while after longer exposure time (24 h) none of the tested EDCs induced genotoxic effect at relevant concentrations found in treated paper mill wastewaters. When evaluating and predicting the genotoxic potential of paper mill wastewaters that are released into the environment, it is very important to account that not only the EDCs are present in such complex water samples but also other pollutants and between all these components synergistic or antagonistic effects may occur.

The literature data regarding the cytotoxic and genotoxic potential of EDCs in eukaryotic assays are very limited. It was shown that DMP did not induce gene mutations in *S. typhimurium* strains TA98, TA100, TA1535, or TA1537, with or without rat and hamster liver S9. In cultured Chinese hamster ovary cells, DMP induced sister chromatid exchanges in the presence of S9, while in the absence of S9 it was negative (NTP 1995). Also in our study DMP was negative in the SOS/umuC assay, while in comet assay it induced DNA strand break formation in HepG2 cells.

NTP (1995) reviewed the published data (seven studies) and reported that DEP may be weakly mutagenic in Salmonella strains TA100 and/or TA1535, which mutate via base substitution. However, because the *in vitro* data were sparse and no *in vivo* data were available for analysis, they considered the mutagenic profile to be incomplete. NTP (1995) then proceeded to conduct additional tests. They reported no mutagenic response with DEP in *S. typhimurium* strains TA98, TA100, TA1535 or TA1537 either with or without rat or hamster liver S9. They also reported no chromosomal aberrations with DEP in Chinese hamster ovary cells with or without rat liver S9. However, DEP induced sister chromatid exchanges at concentrations of 167 to 750 mg/L with, but not without, rat liver S9. In our
study DEP at concentrations of 1 to 1000 µg/L was negative in the SOS/umuC assay, while in comet assay it induced DNA strand break formation in HepG2 cells. NTP (1995) noted that, although the positive sister chromatid exchange test might indicate a potential for \textit{in vivo} DNA damage, this endpoint is highly sensitive and does not correlate well with carcinogenic effects in rodents. Under the conditions of 2-year studies, there was no evidence of carcinogenetic activity of DEP in male or female F344/N rats (NTP 1995). However, there was equivocal evidence of carcinogenetic activity of DEP in male and female B6C3F1 mice based on increased incidences of hepatocellular neoplasms, primarily adenomas (NTP 1995).

International Programme on Chemical Safety (IPCS 1997) reviewed a number of mutagenicity and related endpoints for DBP and concluded that the weight of the evidence indicated that DBP is not genotoxic. Also in our study DBP was negative in the SOS/umuC assay, while in comet assay it induced strand break formation in HepG2 cells.

A recent review by the International Programme on Chemical Safety (IPCS 1999) stated that BBP is not genotoxic. Also in our study BBP was negative in the SOS/umuC assay, while in comet assay it induced DNA strand break formation in HepG2 cells. However, in the available studies, the genotoxic activity of BBP has been week and is often consistent with secondary effects on DNA.

\textit{In vivo} study of the US National Toxicology Program (2000), BEHP was shown to be a hepatocarcinogen in rats and mice (Kluwe et al. 1982). For this reason in 1992 the US Environmental Protection Agency (US-EPA) classified BEHP as a Group B2 (probable human) carcinogen(US-EPA 1999). However, BEHP neither acts as a direct genotoxic agent in standard short-term \textit{in vitro} or \textit{in vivo} assays, nor as an \textit{in vivo} tumor initiator (Budroo and Williams 1993). Therefore, the International Agency for Research on Cancer (IARC) (2000) reclassified BEHP from category 2B (possible carcinogenic to humans) to category 3 (cannot be classified as to its carcinogenicity to humans). \textit{In vivo} studies have shown that BEHP caused hepatocellular lesions of the rat's liver and Leydig cell tumors of the the Sprague–Dawley (SD-CD) rat testes (Voss et al. 2005). Also in our study, BEHP induced strand break formation in HepG2 cells, while it was negative in the SOS/umuC assay.
BPA at concentrations 1 µM induced DNA damage in ER-positive MCF-7 cell line after 3 h of exposure evaluated by comet assay (Iso et al. 2006). When higher concentration of BPA (100 µM) was applied to MCF-7 cells DNA strand break formation was detected already after 1 h of the exposure and increased with time (up to 24 h). Also in our study, BPA at concentrations 43.8 nM and higher induced strand break formation in HepG2 cells. In ER-negative MDA-MB-231 cells only slight induction of DNA damage was detected after 3 and 24 h treatment with 100 µM BPA. Using the γH2AX method, which detects DNA double-strand breaks, BPA increased the formation of γH2AX foci in MCF-7 cells after 3 h of the exposure, while the γH2AX foci were indistinct in ER-negative MDA-MB-231 cells (Iso et al. 2006). Similarly Lee et al. (2003) observed genotoxic activity of BPA in mouse lymphoma cells by using comet assay; however, the authors concluded that the effect was due to cytotoxicity and not genotoxicity.

Rivero et al. (2008) found that NP at concentrations 1-16 µg/L was not genotoxic in comet assay using peripheral erythrocytes of Oreochromis niloticus after 72 h exposure, while Atienzar et al. (2002) reported that NP at concentration 10 µg/L, caused DNA damage in DNA extracted from exposed barnacle larvae detected using the RAPD assay. In our study we also found that NP at concentration 10 µg/L caused DNA damage in HepG2 cells.

Pure EDCs were negative in the SOS/umuC assay, while all induced DNA strand break formation in HepG2 cells indicating that EDCs contributed to genotoxic activity detected in HepG2 cells. In the bacterial assay exogenous liver fraction is added, which contains only CYP enzymes (enzymes involved in the activation of promutagens) responsible for the activities of phase I, while comet assay was performed on the human derived HepG2 cells which have retained the activities of phase I and II enzymes. Phase I enzymes include the cytochromes P450 (functionalize compounds by oxidation, reduction or hydrolysis), whereas phase II enzymes carry out the conjugations of functionalized compounds with endogenous ligands (e.g., glucuronic acid, glutathione and sulfate), that play key roles in the activation and detoxification of DNA reactive carcinogens (Uhl et al. 2000). Our study indicated that human hepatoma HepG2 cells are useful tool for the assessment of DNA damage induced by pure EDCs. The main advantage of comet assay relative to other genotoxicity assays is its high sensitivity for detecting low levels of DNA damage (Tice at al. 2000). Similarly, other authors
also found higher sensitivity of genotoxicity assays using eukaryotic cells in comparison to bacterial assay (West et al. 1988, Fabacher et al. 1988, Chen and White 2004).

Cytotoxic and genotoxic activity of seven EDCs (DMP, DEP, DBP, BBP, BEHP, BPA and NP) were evaluated since they are present and were detected in paper mill wastewaters. Subsequently, these EDCs end up in the environment. All seven EDCs have been shown to produce DNA damage in human hepatoma HepG2 cells in comet assay. However, comet assay is not enough to confirm genotoxic effect of selected EDCs and their potential hazard for humans. For better evaluation and prediction of adverse effects of tested EDCs further in vitro as well as in vivo studies observing different end-points are needed. Regarding the effects of EDCs on the aquatic environment bioassays using aquatic organisms such as Zebrafish, Daphnids, Rainbow trout, Fathead minnow, European eel, Mosquitofish, Japanese medaka test should be used.

The applied combination of cytotoxicity and genotoxicity/mutagenicity testing proved be a useful set of quick and simple bioassays for the detection of potentially dangerous cytotoxic and mutagenic/genotoxic contaminants in un-concentrated water samples. With this study we also confirmed that the cytotoxicity and mutagenicity/genotoxicity bioassays should be an integral tool in the evaluation of cytotoxic and/or mutagenic/genotoxic potential of complex wastewater samples before the release into environment, as well as for the monitoring of surface water quality, providing data useful in risk assessment.
4.4 Different treatment procedures to degrade and/or remove selected endocrine disrupting compounds from paper mill wastewaters

The purpose of this part of the study was to find out, which wastewater treatment method, among biological (anaerobic and aerobic) plus membrane filtration (MBR, UF and RO) trains of treatment, and AOP (Fenton reaction, photo-Fenton reaction, photocatalysis with TiO₂ reagent and ozonation) treatments, is the most effective method for removing and/or degrading DMP, DEP, DBP, BBP, BEHP, BPA, NP and COD from a paper mill effluent of a 100% recovered paper mill. At pilot-scale, two pilot plants (pilot plant A and pilot plant B) were running in parallel and the following treatments were compared: (pilot plant A) anaerobic treatment followed by aerobic treatment, UF and RO; and (pilot plant B) anaerobic treatment followed by MBR and RO. Moreover, at lab-scale, four different AOP were applied.

4.4.1 Removal of endocrine disrupting compounds by biological and membrane treatments

COD is a common parameter used for the characterisation of organic matter present in paper mill effluents (Lacorte et al. 2003; Latorre et al. 2005; Balabanič and Krivograd Klemenčič 2011). COD in paper mill effluents depends on the raw material used, the type of paper produced and type of paper machine (Latorre et al. 2005). For the removal of organic load from paper mill wastewaters, an activated sludge process has been widely used (Pokhrel and Viraraghavan 2004; Balabanič and Krivograd Klemenčič 2011). Although conventional biological WWTPs are not designed to remove traces of organic contaminants as EDCs from wastewater, studies indicate removal efficiencies from 69% to 92% of aerobic, anaerobic or combined anaerobic-aerobic biological WWTPs for removal of selected EDCs from wastewaters (Balabanič and Krivograd Klemenčič 2011; Vethaak et al. 2005; Lagana et al. 2004). In the case of pilot plant A, our results showed that removal efficiencies after first (anaerobic) treatment step were 63% for COD, 64-69% for DMP, DEP, DBP, BBP and BEHP, 68% for BPA and 64% for NP; and after second (aerobic) treatment step by and additionally 75% to 80% for DMP, DEP, DBP, BBP and BEHP, 72% for BPA and 71% for
NP (Figure 17). In the case of the pilot plant B, our results showed that removal efficiencies after first (anaerobic) treatment step were 64% for COD, 65-71% for DMP, DEP, DBP, BBP and BEHP, 69% for BPA and 66% for NP (Figure 18). According to Soares et al. (2008) and Zhao et al. (2008), biodegradation is the dominant mechanism of phthalates, BPA and alkylphenols degradation in water. Vidal and Diez (2005) stated that the majority of EDCs removal from wastewater is by biodegradation, and that primary settling, aerating volatilisation, chemical precipitation and sludge absorption are in the minority. Our results of EDCs degradation regarding activated sludge treatment steps in both pilot plants are in agreement with Balabanič and Krivograd Klemenčič (2011).

Removal efficiencies after UF (third treatment step in the pilot plant A) were 77% for COD, 93% to 97% for DMP, DEP, DBP, BBP and BEHP, 93% for BPA and 93% for NP (Figure 17). High removal efficiencies of EDCs by UF are reported also by Snyder et al. (2003) and Bodzek et al. (2004). Removal efficiencies of EDCs from paper mill wastewaters can be compared with that of EDCs from synthetic aqueous solution. Membrane filtration technologies, such as UF has been shown as a promising alternative for removing of different micro-pollutants (Yoon et al. 2006). Compared to conventional processes, UF's remarkable advantage is the high quality of effluent, including extremely low organic concentration, and the removal of microbes and viruses without chemical disinfection. Physical means may be advantageous for their simple operation and high removal efficiency (Yoon et al. 2006).

Removal efficiencies after MBR (second treatment step in the pilot plant B) were 90% for COD, 95-97% for DMP, DEP, DBP, BBP and BEHP, 90% for BPA and 94% for NP (Figure 18). These values are close to the data obtained with the activated sludge combined with UF. The significant reduction in COD could be attributed to the oxidation reactions due to aeration in combination with activated sludge. Our results also agree with those of Lew et al. (2009), who observed an 88% for COD removal from wastewaters treated by MBR. According to Wintgens et al. (2002) and Clara et al. (2005) MBR could remove more than 80% of potential EDCs from wastewaters, which is also in agreement with our results. Compared to conventional wastewater treatment, the advantage of MBR is the high quality of effluent, including extremely low EDCs concentrations without chemical treatment, better control of biological activity, effluent that is free of bacteria and pathogens, smaller plant size and
higher organic loading rates (Wintgens et al. 2002); however one of the main disadvantages is its energy consumption. Conversely, removal efficiency after MBR was the same as after an aerobic treatment followed by UF, which is less energy-consuming process. Therefore the final definition of the treatment train at the industrial scale must be assessed in economical, environmental and viability terms.

However, we must realise that for organic contaminants such as EDCs, the removal rates achieved in our research with the use of UF and MBR are not sufficient and in the case when the persistent contaminants are released to the receiving natural waters, they will be consequently consumed by aquatic organisms and through them may also enter in human food chain. RO was introduced in both pilot plants as a final treatment stage and in both cases the removal of COD and selected EDCs was 100% (Figure 17 and 18). The significant drawback to be considered with RO are its high energy consumption and the formation of a reject, which may require further treatment. Almost complete removal of EDCs by RO is also reported by Comerton et al. (2008) and Bolong et al. (2009). Removal of contaminants by membrane filtration technologies is a separative process, not like biodegradation or chemical oxidation, hence no by-products or metabolites are newly produced. According to Liu et al. (2009), the rejection efficiency of EDCs by membranes strongly depends on the physico-chemical properties of EDCs, such as molecular weight, octanol/water partition coefficient ($K_{ow}$), water solubility and electrostatic properties. EDCs removal by the membrane filtration technologies is mainly due to size exclusion, charge repulsion and adsorption (Liu et al. 2009).
**Figure 16:** Removal efficiencies of chemical oxygen demand and investigated endocrine-disrupting compounds for different treatments in pilot plant A applied with standard deviations.

An-anaerobic, Aer-aerobic, UF-ultrafiltration, RO-reverse osmosis.

**Figure 17:** Removal efficiencies of chemical oxygen demand and investigated endocrine-disrupting compounds for different treatments in pilot plant B applied with standard deviations.

An-anaerobic, MBR-membrane bioreactor, RO-reverse osmosis.
4.4.2 Laboratory scale advanced oxidation processes

According to IPPC (2001), the general Best Available Technology for pulp and paper industry wastewater treatment are considered: (a) an activated sludge system under the prerequisite that concentrated streams containing non-biodegradable compounds are pre-treated separately; (b) pre-treatment of highly-loaded, selected and segregated single wastewater streams containing non-biodegradable compounds with chemical oxidative treatments and AOPs. AOPs are well known for their capacity for oxidising and mineralising bio-recalcitrant organic compounds (Malato et al. 2002; Katsumata et al. 2004; Gültekin and Ince 2007; Xu et al. 2008). In the frame of our research, four different AOPs were assessed with the intention of discovering which type of AOP treatment is the most effective for the removal of selected EDCs from raw paper mill wastewater of a mill for 100% recovered paper. However, in the treatment of large volumes of wastewater, cost is the most important factor.

4.4.2.1. Fenton reaction

In the treatment of a large amount of wastewater (>10 m$^3$/t), the most important factor concerned for industry is the cost. The major cost in the Fenton treatment is the chemical cost (approx. 10.44 €/m$^3$ wastewater), especially the cost for H$_2$O$_2$. Based on such concerns, the H$_2$O$_2$ dose should be determined properly. In this study, the initial concentration of H$_2$O$_2$ was determined by optimising its molar ratio to COD (COD (mg/L) x 2.125 = mg/L of H$_2$O$_2$). Previous experiments (data not shown) indicated that COD removal is not significantly increased with H$_2$O$_2$ concentrations up to 2.125 x COD (mg/L) and that the COD removal increase (less than 8% for double H$_2$O$_2$ concentration) is not compensated for the increment of cost associated. The maximum duration of the wastewater treatment with the Fenton reaction in our research was 30 min, since we discovered that a 30 min treatment was enough to consume all the H$_2$O$_2$ and reach the maximum degradation of COD and EDCs. The main process variables affecting the rate of Fenton reaction are the reagents H$_2$O$_2$ and Fe$^{2+}$ concentrations and the ratios between them (Kim et al. 1997; Hermosilla et al. 2009b). Increasing the concentration of H$_2$O$_2$ is important to obtain high oxidation efficiencies. If the concentration of one reactant is increased to observe its positive effect, thereby keeping the other one constant, the [H$_2$O$_2$]:[Fe$^{2+}$] molar ratio and hence the oxidation conditions will change significantly (Hermosilla et al. 2009b). As seen in Figure 19A, the degradation rate
increased with increasing initial $\text{H}_2\text{O}_2$ concentration. This is due to more hydroxyl radicals and hydroperoxyl, among other oxidizing species, being produced. According to Kim et al. (1997) and Hermosilla et al. (2009a) $[\text{H}_2\text{O}_2]:[\text{Fe}^{2+}]$ ratio of 1.5 is optimal for COD treatment of landfill leachate, which was confirmed also with our research for paper mill wastewater from a 100% recovered paper mill. Treatment of wastewater with Fenton reaction at $[\text{H}_2\text{O}_2]:[\text{Fe}^{2+}]$ ratio 1.5 and treatment time of 30 min reached the highest degradation of COD and EDCs (75%, 90-91%, 91% and 90%, respectively), followed by $[\text{H}_2\text{O}_2]:[\text{Fe}^{2+}]$ ratio 1.0 (69%, 81-84%, 82% and 80%, respectively) and $[\text{H}_2\text{O}_2]:[\text{Fe}^{2+}]$ ratio 2.5 (63%, 68-75%, 71% and 67%, respectively) (Figure 19A). Sevimli (2005) found 83% COD degradation in the optimal Fenton operation conditions (pH=4, $[\text{H}_2\text{O}_2]$=200 mg/L, $[\text{Fe}^{2+}]$=100 mg/L, t=45 min), however, this effect was observed in our experiment in biological treated paper mill wastewaters. Wongniramaikul et al. (2007) found an 87% degradation of diisobutyl phthalate (DIBP) from synthetic aqueous solution (14 mg/L) in the optimal Fenton operation conditions (pH=3, $[\text{H}_2\text{O}_2]:[\text{Fe}^{2+}]:[\text{DIBP}]$ molar ratio=5:5:1, t=30 min). From Figure 19B, it is evident that removal of COD and EDCs started right after initiation of the reaction and was slowly increasing with time due to the formation of by-product, mainly carboxylic acid which is more resistant to chemical oxidation (Hermosilla et al. 2009b).
Figure 18: Removal efficiencies of chemical oxygen demand and investigated endocrine-disrupting compounds (A) and time intervals (B) for Fenton reaction applied with standard deviations.

4.4.2.2. Photo-Fenton reaction

The Fenton reaction generates sludge, which can be separated from the wastewater, but needs the application of a thickening process with additional operating costs (approx. 36.26 €/m$^3$ wastewater) and it must be properly discarded with additional cost. The use of UV light in combination with the Fenton reagent (photo-Fenton method) re-generates the ferrous iron with a reduction of the ferric form (Kavitha and Palanivelu 2004). According to Hermosilla et al. (2009a), the photo-regeneration of Fe$^{2+}$ from Fe$^{3+}$ allows reducing up to 32 times the quantity of FeSO$_4$ added to run an optimal Fenton treatment, thus reducing the production of iron sludge. Furthermore, the addition of UV-light to Fenton process produces additional hydroxyl radicals via photolysis (Kavitha and Palanivelu 2004). Experiments were performed...
with two different concentrations of H₂O₂ (Figure 20A) in order to attempt to reach a 100% COD reduction. All H₂O₂ was consumed within 60 min in the first trials (COD (mg/L) x 2.125 = mg/L of H₂O₂); degradation efficiency was app. 96% (Figure 20B). Double amounts of H₂O₂ were added in the second trials (COD (mg/L) x 4.250 = mg/L of H₂O₂). All H₂O₂ was consumed within 120 min; degradation efficiency was app. 98% (Figure 20B). The maximum duration of the wastewater treatment with the photo-Fenton reaction was 120 min, since we found that a 120 min treatment was enough to consume all the H₂O₂ and reach the maximum degradation of COD and selected EDCs. According to Hermosilla et al. (2009b) and Ioan et al. (2007), the Fenton reaction is more efficient in the presence of UV light, as UV light may promote photo-decarboxylation of ferric carboxylates and reduce ferric iron yielding additional hydroxyl radicals by photolysis. Also in the case of our study, the photo-Fenton reaction was more efficient in COD and selected EDCs degradation than the conventional Fenton reaction. Our research showed that the treatment of wastewater by adding a double amount of H₂O₂ reached the highest COD and selected EDCs removal efficiencies (98%, 98-99%, 99% and 98%, respectively). Also according to Hermosilla et al. (2009a, 2009b), an increase of COD removal in photo-Fenton treatment is possible by adding extra quantities of H₂O₂, although the cost of the treatment is significantly higher and extra removal of COD and selected EDCs may not be significant. Figure 20C shows the evolution of COD and selected EDCs removal when the H₂O₂ dosages were tested. Ferrous iron regeneration during the photo-Fenton reaction produces the evolution of the Fenton reaction and the addition of an extra amount of H₂O₂ allows continuing the reaction and to achieve a total COD and selected EDCs reduction. After 20 min of treatment, efficiencies reached 39% for COD and 42-50% for selected EDCs. A further 20 min of treatment contributed an additional 32% for COD and 20%-26% for selected EDCs; the following 20 min contributed additional 19% for COD and 16-20% for selected EDCs, while every further 20 min of treatment contributed less to the COD and selected EDCs removal. The last 20 min of treatment contributes only an additional 0.5% for COD, 1-2% for selected EDCs (Figure 20C). These results agree with the literature. Hermosilla et al. (2009b) found the same results (96-99%) for COD degradation (contaminated water) in the optimal photo-Fenton conditions (pH=3, [H₂O₂]=30 mM, [Fe²⁺]=0.8 mM, t=60 min). Yang et al. (2005) observed 75.8% degradation of DEP from synthetic aqueous solution (10 mg/L) in the optimal photo-Fenton conditions (pH=3, [H₂O₂]=47.4 µM/min, [Fe²⁺]=0.167 mM, t=120 min); the reason for which could be higher
initial concentrations of DEP (10 mg/L). Chiou et al. (2006) found a 92% degradation of DBP from a synthetic aqueous solution (5 mg/L) in the optimal photo-Fenton conditions (pH=3, [H$_2$O$_2$]=0.5 mM, [Fe$^{2+}$]=0.45 mM, t=90 min). Gkorgkolia et al. (2009) found a 90% degradation of DBP from synthetic aqueous solution (2000 ppm) in the optimal photo-Fenton conditions ([H$_2$O$_2$]=20 mg/L, [Fe$^{2+}$]=1 mg/L, t=60 min). Katsumata et al. (2004) found >90% degradation of BPA from synthetic aqueous solution (10 mg/L) in the optimal photo-Fenton operation conditions (pH=4, [H$_2$O$_2$]=4 mM, [Fe$^{2+}$]=0.4 mM, t=36 h).
**Figure 19:** Removal efficiencies of investigated endocrine-disrupting compounds (A), chemical oxygen demand (B) and time intervals (C) for photo-Fenton reaction applied with standard deviations.
4.4.2.3. Photocatalysis with TiO$_2$ reagent

Treatment of wastewater with photocatalysis with a TiO$_2$ reagent was performed with an addition of 5g or 10g of powdered TiO$_2$ (Figure 21A) with the intention of discovering at which amount of added powder TiO$_2$, COD and selected EDCs degradation efficiencies were the highest. The measurements of COD and selected EDCs were performed at four different time intervals (0, 60, 120 and 180 min). Treatment of wastewater with an added 10g/L TiO$_2$ reagent and treatment time of 180 min reached the highest degradation of COD and selected EDCs (48%, 73-86%, 80% and 76%, respectively), followed by treatment with added 5g/L TiO$_2$ and treatment time 180 min (38%, 62% to 81%, 71% and 66%, respectively). The results showed that the removal of COD by photocatalysis with TiO$_2$ reagent was poor, while degradation of selected EDCs was higher (Figure 21A). An increase of COD and EDCs degradation in photocatalysis with TiO$_2$ may be possible by adding extra quantities of TiO$_2$ until TiO$_2$ concentration produces a shadow effect that reduces the efficiency, although the cost of the treatment is significantly higher and extra degradation of COD and EDCs may not be significant. As previously reported by different authors (Yeber et al. 2000), photocatalysis treatment may remove toxic and non-biodegradable substances to a great extent, it is also an alternative for pre- or post-biological treatment. Perez et al. (2002) and Rodrigues et al. (2008) applied photocatalysis by TiO$_2$ reagent to paper mill effluents and have obtained reductions of COD between 16% and 40%, which is in accordance with our results. No data exists on the removal of selected EDCs from paper mill wastewaters with photocatalysis by TiO$_2$ reagent. However, Xu et al. (2008) observed an 80% degradation of BBP from synthetic aqueous solution (1 mg/L) in the optimal photocatalysis conditions (pH=7, [TiO$_2$]=2 g/L, t=120 min) and Lau et al. (2005) observed a 100% degradation of DBP from synthetic aqueous solution in 90 min, while Chung and Chen (2009) observed a 40-100% degradation of BEHP from synthetic aqueous solution (25-300 µg/L) in the optimal photocatalysis conditions (pH=4, [TiO$_2$]=100 µg/L, t=150 min). However, a disadvantage of the photocatalysis with TiO$_2$ reagent is the relatively high energy consumption and cost of TiO$_2$ (total cost of wastewater treatment is approx. 31.90 €/m$^3$ wastewater).
Figure 20: Removal efficiencies of investigated endocrine-disrupting compounds, chemical oxygen demand (A) and time intervals (B) for photocatalysis with TiO$_2$ reagent applied with standard deviations.
4.4.2.4. Ozonation

The paper mill effluent was exposed to ozone or ozone + H₂O₂ oxidation to determine their effectiveness for COD and selected EDCs degradation. The measurements of COD and selected EDCs were performed at four different time intervals (0, 60, 120 and 180 min). As can be seen from the Figure 22A, limited COD and selected EDCs degradation rates were obtained. COD and selected EDCs degradation within first 60 min were significant, but the oxidation rate decreased as the reaction proceeded (Figure 22B). Treatment of wastewater with added ozone with 50 mM H₂O₂ and an added treatment time of 180 min achieved the highest degradation of COD, DMP, DEP, DBP, BBP, BEHP, BPA and NP (59%, 90-92%, 91% and 92%, respectively), followed by treatment with ozone only and a treatment time of 180 min (38%, 74-79%, 78% and 76%, respectively), and treatment with added ozone with 50 mM H₂O₂ and a treatment time of 120 min (45%, 74-75%, 75% and 75%, respectively) (Figure 22A). The results showed that the removal of COD by ozone only and ozone with an added 50 mM H₂O₂ was poor, while removal of DMP, DEP, DBP, BBP, BEHP, BPA and NP was higher. As with photocatalysis, ozone may remove toxic compounds and it may be used as pre- and post-biological treatment step. Sevimli et al. (2005) applied ozonation to paper mill effluents and obtained COD removal of 61-64%, which is more or less in agreement with our results. The influence of the addition of H₂O₂ to the wastewater sample for the COD and DMP, DEP, DBP, BBP, BEHP, BPA and NP degradation was low. A major disadvantage of the ozonation is the relatively high cost of ozone generation (approx. 35.64 €/m³ wastewater) coupled with the short half-life ozone period.
Figure 21: Removal efficiencies of investigated endocrine-disrupting compounds, chemical oxygen demand (A) and time intervals (B) for ozonation applied with standard deviations.

4.4.3 Costs of investigated wastewater treatment processes

Each of the investigated Slovenian paper mills produce around 2x10^6 m³ wastewater each year. Paper mill wastewaters contain suspended solids (mainly fibres), lignin and its derivates, fatty and resin acids, tannins and many more, making it difficult to treat. The overall costs are represented by the sum of the investment costs, maintenance cost and the operating costs (Tab. 8). The investment costs for aerobic treatment plant is app. 4.500.000 € (personal communication from mill manager). The costs of electricity and reagents being those shown in Table 9. The costs per g of COD reduction were calculated according to the % of COD reduction. According to the calculation of installing, maintance, energy consumption and
reagents cost, we found out that anaerobic wastewater treatment is the most cost effective wastewater treatment procedure. The relative costs for paper mill wastewater treatment are the highest for ozonation followed photocatalysis with TiO$_2$ reagent, ozonation + hydrogen peroxide, photo-Fenton reaction, RO, Fenton reaction, MBR, UF, aerobic treatment and anaerobic treatment. Prices converted a 10 year operation for investigated Slovenian paper industry are: anaerobic wastewater treatment (app. 500.000 €), aerobic wastewater treatment (app. 750.000 €), filtration with UF (app. 1.250.000 €), MBR (app. 1.500.000 €), Fenton reaction (app. 2.000.000 €), RO (app. 2.500.000 €), photocatalysis with TiO$_2$ reagent (app. 4.500.000 €), ozonation (app. 5.500.000 €), photo-Fenton reaction (app. 5.750.000 €) and ozonation + hydrogen peroxide (app. 8.000.000 €) per each year. Economical cost assessment of tested advanced oxidation processes at laboratory scale are present in Table 9.

**Table 8**: Economical cost assessment of tested aerobic and anaerobic biological treatment and membrane filtrations.

<table>
<thead>
<tr>
<th></th>
<th>Aerobic treatment</th>
<th>Anaerobic treatment</th>
<th>Membrane bioreactor</th>
<th>Ultrafiltration</th>
<th>Reverse osmosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Investment cost*</td>
<td>4.500.000 €</td>
<td>5.000.000 €</td>
<td>300.000 €</td>
<td>200.000 €</td>
<td>250.000 €</td>
</tr>
<tr>
<td>Maintenance cost per year*</td>
<td>300.000 €</td>
<td>0 € **</td>
<td>1.450.000 €</td>
<td>1.200.000 €</td>
<td>2.250.000 €</td>
</tr>
<tr>
<td>Operating cost per year*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cost (€/m$^3$)</td>
<td>0.32</td>
<td>0.21</td>
<td>0.61</td>
<td>0.50</td>
<td>0.95</td>
</tr>
</tbody>
</table>

* personal communication from mill managers.
** maintenance cost and operating cost are compensated by green energy production.

**Table 9**: Economical cost assessment of tested advanced oxidation processes at laboratory scale (Fenton process: $[\text{H}_2\text{O}_2] / [\text{Fe}^{2+}] = 1.5, [\text{H}_2\text{O}_2] / \text{COD} = 2.125$; Photo-Fenton: $[\text{H}_2\text{O}_2]$
/ [Fe$^{2+}$] = 32 [H$_2$O$_2$] / COD = 2.125; Photocatalysis: [TiO$_2$] = 10 g/L, time=120 minutes; O$_3$; time = 180 minutes; O$_3$/H$_2$O$_2$: [H$_2$O$_2$] = 50 mM, time = 180 minutes).

<table>
<thead>
<tr>
<th></th>
<th>Fenton</th>
<th>Photo-Fenton</th>
<th>Photocatalysis</th>
<th>O$_3$/H$_2$O$_2$</th>
<th>O$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy consumption</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(kWh)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TiO$_2$ (kg)</td>
<td></td>
<td></td>
<td></td>
<td>1*</td>
<td></td>
</tr>
<tr>
<td>FeSO$_4$$\cdot$7H$_2$O (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$O$_2$ (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cost (€/m$^3$)†</td>
<td>10.44</td>
<td>36.26</td>
<td>31.90</td>
<td>38.07</td>
<td>35.64</td>
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<tr>
<td>% COD removal</td>
<td>75</td>
<td>96</td>
<td>39</td>
<td>59</td>
<td>38</td>
</tr>
<tr>
<td>€/g COD removal †</td>
<td>0.007</td>
<td>0.018</td>
<td>0.039</td>
<td>0.031</td>
<td>0.045</td>
</tr>
</tbody>
</table>

* Considering a 10% catalyst replacement.
† The price of chemicals was taken from the Chemical Market Reporter; and energy price was 0.066 €/kWh (personal communication from mill managers).

The use of biological treatment is attractive due to its low operating cost, but the retention time is very high relative to that of other processes. On the other hand, the removal rate of AOPs is relatively high while the operating cost is relatively expensive due to the use of reagents and irradiation source. The fixed/capital cost of AOPs is also higher as compared to biological methods. Capital and operation costs of biological treatment are 3-20 times cheaper than those of AOPs, respectively (Marco et al. 1997; Mamma et al. 2004; Poole 2004). Based on the cheaper construction and their operating cost, it is desirable to maximize the retention time and the removal rates of contaminants in biological processes. AOPs for wastewater treatment are not an economical process due to their high operating cost. Thus, it is suggested to integrate these technologies with other post-treatment methods such as biological processes. However, the optimal solution (totally reduced EDCs from Slovenian paper mill wastewaters) with biologically treatment, filtration and AOPs, cost app. 1.000.000 € per each year.
5. CONCLUSIONS

The paper industry is one of the largest industrial polluter in terms of wastewater volumes produced and organic discharge. Paper mill wastewaters often contain harmful contaminants such as endocrine disrupting (EDCs) and other genotoxic compounds (2,2-dibromo-3-nitrilopropionamide, pentachlorophenol, resin acids, fatty acids and many more). Emission of EDCs and genotoxic compounds are becoming more and more important, but the lack of data is striking. Our results indicated that DMP, DEP, DBP, BBP, BEHP, BPA and NP were present in raw and biologically treated paper mill effluents from both Slovenian paper mills with different production processes (paper mill A is manufacturing label papers and flexible packaging papers from fresh cellulose fibres; paper mill B is manufacturing carton board from 100% recovered fibres). On-site installed conventional aerobic and combined anaerobic-aerobic biological wastewater treatment plants are efficient for removing of phthalates, bisphenol A and nonylphenol from 54-98%; however for substances such are investigated compounds this is not enough. As phthalates, bisphenol A and nonylphenol are detrimental already if present in low concentration levels they should be seriously considered and their impact further studied.

The results obtained in the present study demonstrate that two third of the untreated paper mill wastewaters from paper mill B were genotoxic in comet assay. More than half of the untreated paper mill wastewaters from paper mill B were mutagenic in Ames MP\textsuperscript{TM} 98/100 Aqua assay. This effect was not found in untreated paper mill wastewaters from paper mill A, which use virgin fibres in paper production processes. In the study we confirmed that comet test is a very sensitive method for genotoxicity screening of un-concentrated wastewater samples. The applied combination of cytotoxicity, mutagenicity and genotoxicity testing was proven to be a useful combination of simple tests for the detection of potentially dangerous cytotoxic and mutagenic/genotoxic contaminants in native water samples. In this study we also confirmed that the bioassays should be an integral tool in the evaluation of toxic potential of complex wastewater samples before the release into environment, as well as for monitoring of industrial effluents and surface water quality, providing data useful for risk assessment. It was also confirmed that comet assay is a very sensitive method for genotoxic screening of pore EDCs and their artificial mixtures. The applied combination of cytotoxicity and
mutagenicity/genotoxicity testing proved be a useful combination of simple tests for the detection of potentially dangerous cytotoxic and mutagenic/genotoxic compounds.

The efficiently assess the presence of genotoxic compounds in the wastewater, aside from conventional chemical analysis, genotoxicity assays should be included as additional parameters in water quality monitoring programs. Genotoxic parameters, therefore, have been proven to be sensitive and reliable tools in the detection of genotoxic activity in the aquatic environment and thus currently the most valuable biomarkers for ecological risk assessment. Human health and environmental quality risk associated with the presence of phthalates, bisphenol A and nonylphenol in industrial effluents necessitate the utilization of better methods for their efficient reduction.

The treatment of the paper mill effluents has been widely assessed in terms of general contamination load, while little is known about the removal of EDCs. In the present study, the treatment performance of different wastewater treatment procedures at pilot and lab-scale for the reduction of COD and DMP, DEP, DBP, BBP, BEHP, BPA and NP from 100% recovered paper mill wastewaters were investigated. Our study shows that the combination of biological treatment and membrane filtration is very efficient for COD and selected EDCs removal from paper mill wastewaters. Among the selected wastewater treatment methods: RO, photo-Fenton reaction and membrane bioreactor were the most efficient for COD and selected EDCs removal, while the Fenton reaction, photocatalysis with TiO$_2$ and ozonation were less effective. The EDCs degradation from paper mill effluents is a process with high energy consumption, in which cost and efficiency are the key considerations for their application. RO as a final step in both pilot plants provided 100% removal for COD and selected EDCs, but the higher implied energy consumption is an important disadvantage to be considered. The process costs may be also considered the main reason that serious doubts hinder the commercial application of the photo-Fenton process. Biological treatment has proven to be the most cost effective processes (approx. 0.21 €/m$^3$ wastewater for anaerobic treatment and approx. 0.32 €/m$^3$ wastewater for aerobic treatment). However, its removal efficiency for substances such as EDCs is not high enough.
6. SUMMARY

High water usage in pulp and paper industry, results in large amount of wastewater generation. They are also significant contributors of pollutant discharges to the environment. My PhD research was divided into three parts. The first part of PhD research was to determine the level of dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), bis(2-ethylhexyl) phthalate (BEHP), bisphenol A (BPA) and nonylphenol (NP) in paper mill wastewaters from two paper mills with different production processes (paper mill A uses virgin fibres and paper mill B uses recovered fibres as primary source for paper production). These compounds were chosen based on the requirements of the EU Water Framework Directive (2000/60/EC) and Directive (2008/105/EC), which will enter into force in 2015. BEHP, BPA and NP are already on the list of priority substances in EU Water Framework Directive (2000/60/EC) and Directive (2008/105/EC). DMP, DEP, DBP, and BBP were chosen as it is well known from the literature that they may occur in the effluents of paper industry. Target compounds were determined by employing solid phase extraction, followed by derivatization with N,O-bis(trimethyl-silyl)trifluoroacetamide (BSTFA) and gas chromatography-mass spectrometry (GC-MS). The chemical analysis revealed that among seven target compounds, all seven were confirmed in effluents from two paper mills with different production processes. Despite relative high degradation efficiency of biological wastewater treatment plants for target compounds, the concentrations of BEHP and NP in biologically treated paper mill effluents exceeded limits of 1.3 µg/L and 0.3 µg/L according to EU Water Framework Directive (2000/60/EC) and Directive (2008/105/EC).

The second research goal was to assess genotoxic activity of paper mill wastewater and associated surface water samples in which DMP, DEP, DBP, BBP, BEHP, BPA and NP were determined. The mutagenicity/genotoxicity was determined using the combination of standard bacterial test systems – SOS/umuC assay and bacterial assay for reverse mutations – Ames MPF™ 98/100 Aqua assay as well as using genotoxicity test with cell model of human hepatoma HepG2 cells. The genotoxic potential of water samples was evaluated using comet assay that was applied for testing un-concentrated environmental samples. The genotoxic activity of pure and artificial mixtures of target compounds were determined using the combination of standard bacterial test – SOS/umuC test and genotoxicity test with human
hepatoma HepG2 cells. These chemicals were chosen as they occurred in wastewater samples from both Slovenian paper mills. The concentrations of pure EDCs and in artificial mixtures were in ranged from 1 to 1000 µg/L as these concentration were detected in our samples. Of 63 analysed water samples, no one was cytotoxic, mutagenic and/or genotoxic in bacterial SOS/umuC assay. The mutagenic activity of un-concentrated untreated and treated samples of wastewater from paper mill A and associated surface waters were not significantly affected. However, more than a half untreated samples from paper mill B wastewater samples showed mutagenic effects in the Ames MPFTM 98/100 Aqua assay with *S. typhimurium* strains TA100 with metabolic activation S9. This mutagenic effect was not observed after biologically treatment. The viability of HepG2 cells exposed to paper mill wastewaters for 24 h was not significantly affected. The genotoxic activity of un-concentrated untreated and treated samples of wastewater from paper mill A and associated surface waters was not significantly affected. However, two third of the untreated paper mill B wastewater samples showed genotoxic effects in comet assay. All samples from paper mill B after first biological treatment step (aerobic treatment) showed genotoxic activity in HepG2 cells using comet assay. Next step of paper mill B wastewater treatment process was anaerobic treatment. Two third samples after anaerobic treatment were positive. The results obtained for associated surface waters showed that samples sampled up-stream from the place where the effluent flows to the river were negative, with an exception of the sample taken on Wednesday, while more than two thirds (Monday, Wednesday, Thursday, Friday and Saturday) of the surface water samples from down-stream increased the formation of DNA strand breaks in HepG2 cells. When testing pure EDCs in concentration range detected in paper mill wastewater samples no induction of SOS response in *Salmonella typhimurium* TA1535 pSK1002 was detected. The viability of HepG2 cells exposed to 0, 1, 10, 100 and 1000 µg/L of pure DMP, DEP, DBP, BBP, BEHP, BPA and NP for 4 and 24 h was not significantly affected. In HepG2 cells DMP, DEP, DBP, BBP, BEHP, BPA and NP increased % of tail DNA at concentrations from 1-1000 µg/L. DNA damage can be observed already after 4 h treatment and DNA damage also remaining after 24 h treatment. In the study we confirmed that comet test was shown to be a very sensitive method for genotoxicity screening of un-concentrated wastewater samples and pure EDCs. The applied combination of cytotoxicity AND mutagenicity/genotoxicity testing was proven to be a useful combination of simple tests for
the detection of potentially dangerous cytotoxic and mutagenic/genotoxic contaminants in wastewaters as well as native environmental water samples.

The third research goal was to explore, which wastewater treatment procedures are more efficient to degrade and/or remove target compounds from paper mill wastewaters applying the chemical analysis before and after various treatment procedures. Different treatments were compared in their treatment efficiency for removal of DMP, DEP, DBP, BBP, BEHP, BPA, NP and chemical oxygen demand (COD) from collected paper mill wastewater, comprising anaerobic treatment, activated sludge, membrane bioreactor (MBR), ultrafiltration (UF) and reverse osmosis (RO) at a pilot plant scale; and Fenton reaction, photo-Fenton reaction, photocatalysis with TiO$_2$ and ozonation at a laboratory scale. The results show that anaerobic treatments removed DMP, DEP, DBP, BBP, BEHP, BPA and NP in the same percentage that COD (60-70%), while aerobic treatments removed these compounds in an 80%, a 20% more than the COD removed. An UF step such as in MBR treatments removed target compounds in almost a 100%. Advanced oxidation processes obtained a high removal of DMP, DEP, DBP, BBP, BEHP, BPA and NP between 75 to 99% in all the tested treatments. Photocatalysis with TiO$_2$ obtained the worst results and photo-Fenton got almost a 100% of removal the same as COD. Ozone was not so effective than Fenton processes for removing COD but it removed EDCs in a 90%. According to the efficiencies of different paper mill wastewater treatments, calculations of approximately costs were made, and on this basis we can conclude that biological treatment (anaerobic + aerobic) followed by filtration and AOPs was the most affordable and environmentally acceptable.
POVZETEK

Papirna industrija je ena od večjih porabnic vode, ki ima za posledico tudi velike količine odpadnih voda, ki znatno prispevajo k onesnaževanju okolja. Doktorsko delo je razdeljeno na tri dele. V prvem delu smo ugotavljali prisotnost dimetil ftalata (DMP), dietil ftalata (DEP), dibutil ftalata (DBP), benzil butil ftalata (BBP), bis(2-etilheksil) ftalata (BEHP), bisfenol A (BPA) in nonilfenola (NP) v odpadnih vodah dveh papirnic z različnima proizvodnima procesoma (papirnica A za proizvodnjo papirnih izdelkov uporablja primarna lesna vlakna; papirnica B pa uporablja reciklirana vlakna kot primarni vir za proizvodnjo papirnih izdelkov). DMP, DEP, DBP, BBP, BEHP, BPA in NP so bili izbrani na podlagi zahtev Okvirne vodne direktive EU (2000/60/ES) in Direktive (2008/105/ES), ki bosta stopili v veljavo leta 2015. BEHP, BPA in NP so že na seznamu prednostnih snovi v Okvirni vodni direktivi EU (2000/60/ES) in Direktivi (2008/105/ES). DMP, DEP, DBP in BBP pa so bili izbrani na podlagi literature, ki opisuje, da so te snovi lahko prisotne v odpadnih vodah papirne industrije. Spojine so bile zaznane z uporabo ekstrakcije na trdni fazi (SPE), ki ji sledi derivatizacija z N,O-bis (trimetil-silil) trifluoroacetamid (BSTFA) in plinsko kromatografijo z masno spektrometrijo (GC-MS). GC-MS analiza je pokazala, da je med sedmimi izbranimi spojinami, bilo vseh sedem prisotnih v odpadnih vodah iz obeh analiziranih papirnicah. Kljub relativno visoko učinkovitost razgradnje teh snovi na bioloških čistilnih naprav obeh papirnic, so koncentracije BEHP in NP v biološko očiščenih odpadnih vodah presegale mejne vrednosti v višini 1.3 µg/L za BEHP in 0.3 µg/L za NP v skladu z Okvirno vodno direktivo EU (2000/60/ES) in Direktivo (2008/105/ES).

V drugem delu raziskave so bili ponovno vzeti vzorci odpadnih vod iz zgoraj omenjenima papirnica industrijama. V teh vzorcih smo kemično določili prisotnost DMP, DEP, DBP, BBP, BEHP, BPA in NP. Na teh istih vzorcih smo tudi testirali morebitno mutageno/genotoksično delovanja. Mutagenost/genotoksičnost je bila določena s kombinacijo standardnih bakterijskih testov - SOS/umuC testom in testom za bakterijske reverzne mutacije - Ames MPF ™ 98/100 test. Test genotoksičnosti smo izvedli tudi z modelom celic človeškega izvora (HepG2 celice). Genotoksičnost vzorcev vode je bila ocenjena s komet testom, ki je bil prirejen za testiranje kompleksnih okoljskih vzorcev. V nadaljevanju smo naredili še teste mutagenosti/genotoksičnosti čistih kemikalij (DMP, DEP, DBP, BBP, BEHP,
BPA in NP) in njihovih umetnih mešanic v koncentracijah, ki so bile zaznane v vzorcih odpadnih vod obej papirnic. Tudi te teste smo naredili s kombinacijo standardnih bakterijskih testov - SOS/umuC test in test genotoksičnosti s HepG2 celicami. Te kemikalije so bile izbrane, ker bo bile zaznane v analiziranih vzorcih odpadnih vod iz obej slovenskih papirnic. Koncentracije čistih DMP, DEP, DBP, BBP, BEHP, BPA in NP in njenih mešanic so bile v razponu od 1 do 1000 µg/L. Te koncentracije so bile zaznane tudi v naših vzorcih odpadnih vod. Od 63 analiziranih vzorcih odpadnih vode, noben ni bil citotoksičen, mutagen in/ali genotoksičen v bakterijskem SOS/umuC testu. Mutagenska aktivnost kompleksnih vzorcev odpadnih vod iz papirnice A pred in po biološkem čiščenju in s tem povezanih površinskih vod, ni bila izražena. Vendar pa je več kot polovica biološko neobdelanih vzorcev odpadnih vod iz papirnice B pokazalo mutagene učinke v bakterijskem Ames MPFTM 98/100 Aqua testu s sevi Salmonella typhimurium TA100 z dodatkom metabolne aktivnosti S9. Po biološkem čiščenju so bili mutageni učinki izničeni. Preživetje HepG2 celic, ki so bile 24 ur izpostavljene 30% odpadnih vod papirnic ni bilo prizadeto. Komet test ni pokazal genotoksičnega učinka na odpadnih vodah iz papirnice A in z njo povezana površinsko vodo v katero se iztekajo odpadne vode omenjene papirnice. Vendar pa je bilo več kot dve tretjini biološko neobdelanih vzorcev iz papirnice B pozitivnih v komet testu. Po prvem biološkem čiščenju (aerobno čiščenje) so bili vsi vzorci pozitivni v komet testu. Po drugem biološkem čiščenju (anaerobno čiščenje) pa je bilo še vedno več kot dve tretjini vzorcev pozitivnih v komet testu. Rezultati vzorcev površinske vode, ki so bili odvzeti gor-vodno (pred izpustom odpadnih vod) in z njo povezana površinsko vodo, izjemo vzorca, ki je bil odvzet v sredo, niso pokazali genotoksičnega učinka na HepG2 celicah. Vendar pa je bilo več kot dve tretjini vzorcev, ki so bile odvzeti dol-vodno (po izpustu odpadne vode v površinsko vodo) pozitivnih v komet testu, kar kaže na vpliv papirne industrije na okolje. Koncentracije spojin (DMP, DEP, DBP, BBP, BEHP, BPA in NP), ki so bile zaznane v odpadnih vodah obej papirnic, niso pokazale citotoksičnega ali genotoksičnega učinka v SOS/umuC testu. Preživetje HepG2, ki so bile 4 in 24 ur izpostavljene koncentracijam 0, 1, 10, 100 in 1000 µg/L čistega DMP, DEP, DBP, BBP, BEHP, BPA in NP ni bilo prizadeto. Poškodbe DNA so bile opazne že po 4-urni izpostavljenosti in so bile vidne tudi po 24-urni izpostavljenosti izbanim spojinam. V raziskavi je bilo potrjeno, da je komet test zelo občutljiva metoda za testiranje genotoksične aktivnosti kompleksnih okoljskih vzorcev. Uporabljena kombinacija testov citotoksičnosti, mutagenosti in genotoksičnosti se je izkazala kot uporabna kombinacija preprostih in hitrih.
testov za odkrivanje potencialno nevarnih citotoksičnih, mutagenih in genotoksičnih onesnaževalcev v kompleksnih okoljskih vzorcih vod.

Tretji raziskovalni cilj je bilo raziskati, kateri postopki čiščenja odpadnih vod iz papirne industrije so najbolj učinkoviti za razgradnjo in/ali odstranjevanje DMP, DEP, DBP, BBP, BEHP, BPA in NP iz odpadnih vod papirne industrije. Primerjani so bili različni postopki za razgradnjo in/ali odstranjevanje DMP, DEP, DBP, BBP, BEHP, BPA, NP in kemijske potrebe po kisiku (KPK) iz odpadnih vod papirnice. Primerjani so bili: anaerobno čiščenje, aerobno čiščenje, membranski bioreaktor (MBR), ultrafiltracija (UF) in reverzna osmoza (RO) na pilotni napravi, ter Fentonova reakcija, foto-Fentonova reakcija, fotokataliza s TiO₂ in ozonizacija na laboratorijskem nivoju. Rezultati so pokazali, da je anaerobna razgradnja DMP, DEP, DBP, BBP, BEHP, BPA in NP enaka zmanjšanju KPK (60-70%), medtem ko aerobna obdelava razgradi okoli 80% DMP, DEP, DBP, BBP, BEHP, BPA in NP. UF in MBR skoraj popolno zmanjšata koncentracije izbranih snovi. Napredni oksidacijski postopki zmanjšajo koncentracije DMP, DEP, DBP, BBP, BEHP, BPA, NP med 75 do 99%. Med izbranimi metodami je fotokataliza s TiO₂ prikazala najslabše rezultate, medtem ko je foto-Fentonova reakcija skoraj popolna zmanjšala koncentracije DMP, DEP, DBP, BBP, BEHP, BPA, NP in KPK. Ozoniranje ni tako učinkovito za zmanjšanje KPK kot je Fentonova reakcija, vendar pa zmanjša koncentracije DMP, DEP, DBP, BBP, BEHP, BPA in NP za 90%. Glede na učinkovitost različnih postopkov čiščenja odpadnih vod papirne industrije, so bili narejeni izračuni stroškov izgradnje, vzdrževanja in uporabe posameznega čiščenja. Na podlagi izračunov in učinkovitosti lahko sklepmo, da je najcenejše in okolju prijazno biološko čiščenje (anaerobni + aerobno), ki mu sledi filtriranje in napredne oksidacijske metode.
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