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Assessment of Water Mutagenicity

Bachelor thesis

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Bachelor thesis

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Annotation

Since severe health and environmental problems are caused by mutagenic organic pollutants there are urgent needs for the determination of the mutagenicity of surface waters. This work summarizes the literature regarding the occurrence and determination techniques of organic pollutants with mutagenic potential. Additionally, some selected organic pollutants were quantified in surface waters. For pre-concentration Blue Rayon, a sorbent based on a copper phthalocyanine dye suitable for the concentration of polycyclic aromatic compounds with mutagenic potential, was used.

Anotace

Vzhledem k některým vážným zdravotním a environmentálním problémům způsobeným mutagenními organickými polutanty je žádoucí stanovovat mutagenitu povrchových vod. Tato práce shrnuje literaturu zabývající se výskytem a technikami stanovení organických polutantů s mutagenním potenciálem. Navíc byly nalezeny vybrané organické polutanty v environmentálních vodách. Pro zakoncentrování bylo použito modré hedvábí, což je sorbent na bázi ftalocyaninu mědi vhodný pro zakoncentrování polycyklických aromatických sloučenin s mutagenním potenciálem.

Anmerkung

Auf Grund ernster Gesundheits- und Umweltprobleme, welche durch mutagene organische Schadstoffe verursacht werden, ist die Bestimmung der Mutagenität von Oberflächengewässern dringlich erforderlich. Die vorliegende Arbeit fasst die Literatur bezüglich dem Vorkommen und der Bestimmung von mutagenen, organischen Schadstoffen zusammen. Weiters wurden einige ausgewählte organische Schadstoffe in Wasserproben von diversen Oberflächengewässern quantifiziert. Für die Prä-Konzentration wurde „Blue Rayon“, ein Sorbens basierend auf einem Kupfer-Phthalocyanin Farbstoff, welcher für die

Konzentration von polyzyklischen aromatischen Schadstoffen geeignet ist, verwendet.

Declaration

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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Acknowledgement

I would like to acknowledge my supervisor Prof. Ing. Jan Tříska, CSc., who gave me the opportunity to work on such an interesting field of research and who was willing to share his knowledge with me. I also want to thank Ing. Martin Moos, PhD., who was a very wise and patient tutor in the laboratory and also managed organizational stuff for me.

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Last but not least my thanks go to my study colleagues, who turned into cooperating friends during the study, my other friends, and my family for their support whenever I needed them.

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Abbreviations

AαC = amino-carbolines

BaP = benzo[a]pyrene

BaPMO = benzo[a]pyrene monooxygenase

cpt = copper phthalocyaninetrisulfonate

CV = coefficient of variation

din gene = damage-inducible gene

HCA = heterocyclic amines

HPLC = high-performance liquid chromatography

IQ = imidazo-quinoline

lacZ = lac stands for the ability to use lactose, Z indicates the specific gene

MFO = mixed function oxidase

MI = mitotic indices

MR = mutagenicity ratio

MS = mass spectrometry

PAH = polycyclic aromatic hydrocarbons

PCB = polychlorinated biphenyls

PCDD/F = polychlorinated dibenzodioxins/furans

TLC = thin layer chromatography

TWA = time-weighted average

UHPLC = ultra-high-performance liquid chromatography

1. Introduction

As the industry, the agriculture and the households produce a lot of wastewaters that contain a lot of mutagenic and carcinogenic compounds most of the rivers are polluted. The toxic release inventory total surface water discharges and total air emissions for all chemicals by the industry in the United States in the year 2001 are summarized in Table 1. (T. Ohe et al., 2004)

Serious public health and aquatic ecosystem problems might arise from the contamination of surface waters. (Britvić et al., 2010)

Therefore, it is useful to detect mutagenic compounds to be consequently able to inform the public, to protect the nature from them and in the best case to avoid them from being released.

The following bachelor thesis will be based on former research results already reported in the literature and on the evaluation of the current situation by the analysis of the Danube at three locations in Austria, namely Engelhartszell, Linz and Vienna. As reference, water from the barrier lake Mrhal located close to České Budějovice, which is assumed to be very pure, is taken for analysis. For another comparison water samples from Soběslav are analyzed.

For pre-concentration Blue Rayon is used. Especially PAHs including nitro- and amino-derivatives as well as HCAs are adsorbed to it, later desorbed with methanol and further analysed in the laboratory.

Table 1: Industrial discharge in the US in 2001

Industry type	Total water releases ($\times 10^3$ kg)	Total air emissions ($\times 10^3$ kg)
Chemical and allied products	26117.1	103348.6
Food and related products	25018.2	25463.3
Primary metal smelting and processing	20262.5	26132.9
Petroleum refining and related industries	7752.9	21849.6
Paper and allied products	7500.9	71283.5
Electric, gas, and sanitary services	1596.5	325492.4
Electronic and other electrical equipment	1332.2	5770.3
Fabricated metal products	790.8	18346.9
Photographic, medical, and optical goods	646.1	3250.9
Coal mining and coal mine services	344.8	348.7
Tobacco products	241.7	1130.3
Metal mining (e.g., Fe, Cu, Pb, Zn, Au, Ag)	193.8	1294.8
Transportation equipment manufacture	89.9	30251.4
Textile mill products	79.6	2603.9
Stone, clay, glass, and concrete products	73.5	14181.8
Leather and leather products	56.6	547.7
Plastic and rubber products	32.2	34973.1
Solvent recovery operations (under RCRA[b])	10.7	442.0
Lumber and wood products	9.0	13825.1
Industrial and commercial machinery	8.2	3755.7
Petroleum bulk stations and terminals	5.1	9600.4
Chemical wholesalers	0.8	569.0
Furniture and fixtures	0.3	3548.9
Printing, publishing, and related industries	0.1	8750.2
Apparel	<0.1	155.7
No reported SIC code	483.2	1528.3
Miscellaneous manufacturing	16.6	3068.5
Total	100153.0	761763.6

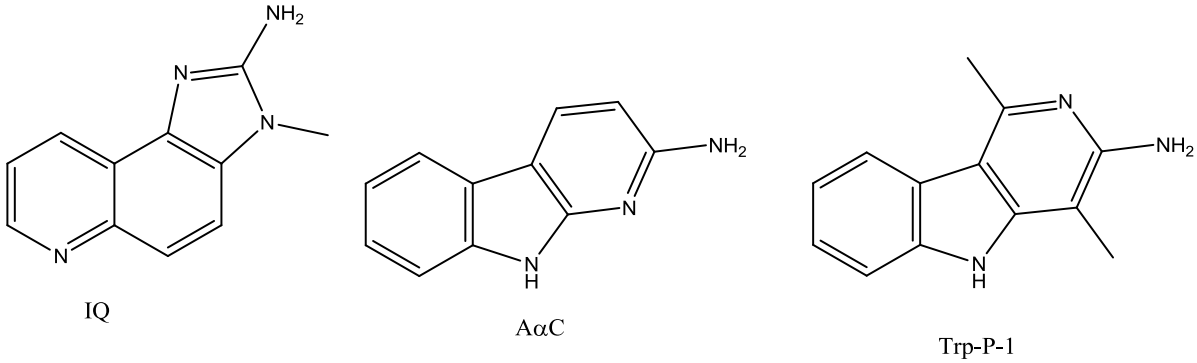
[a] http://www.epa.gov/triexplorer/industry.htm.
[b] The US Resource Conservation and Recovery Act.

2. Sources of mutagenicity in surface waters

A comparison of the mutagenicity of several European and Asian rivers as well as the Canadian St. Lawrence River revealed a strong correlation between human activities and observed genotoxicity (White and Rasmussen, 1998).

Using Blue Rayon as sorbent for pre-concentration 26% of the mutagenicity in Austrian rivers was attributed to the heterocyclic amines IQ, Trp-P-1 and AαC (Kataoke et al., 2000).The chemical structures of these heterocyclic amines are depicted in Figure 1 (J.Bång et al., 2002).

Figure 1: Chemical structures and common names for some HAs



The São Paulo State Water Monitoring Program concentrated on the screening of the genotoxic contamination of Cristais River and the evaluation of possible sources of it.

Monitored were two industries, a galvanizer facility that chemically treats its wastewater and a dye processing plant that utilizes disperse and acid azo-dyes.

While no mutagenic activity was detected in the analysis of the effluent of the galvanizer facility, the dye processing plant effluents showed mutagenicity deemed high according to Houk's effluent classification (Houk, 1992) in both, the treated and the untreated effluent water samples.

Numerous studies concluded that the textile processing industry releases mutagenic wastewater (Sanchez et al., 1988; Coelhoet et al., 1992; Houk, 1992; Claxton, 1997). The treatment of the effluent of a dye factory by an activated sludge system showed to have a limited effectiveness on lowering the mutagenicity. Taking into account the mutagenicity of azo dyes (Venturini and Tamaro, 1979; Reid et al., 1984; Joachim et al., 1985) and their resistance to activated sludge treatment (Pagga and Brown, 1986; Churchley, 1994), they are suspected to be causing the mutagenicity of the industrial effluent. Since arylamines have been condemned as highly mutagenic or even carcinogenic (IARC, 1975) and several breakdown products of the azo dyes are of this kind, aromatic amines were supposed to be the reason for the detected mutagenicity (Shiozawa et al., 1999; Ono et al., 2000; Nukaya et al., 2001).

In Germany the application of plenty of azo dyes that are decomposed to aromatic amines, which are suspicious carcinogens, is prohibited (European Community, 1999).

Also numerous other countries control the aromatic amines (e.g., aniline, benzidine, *m*-chloroaniline, 3,3-dichlorobenzidine, and nitrosoamines) in surface drinking waters (United

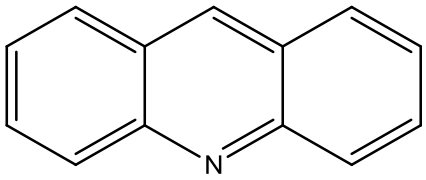
Nations University, 2003). In case azo dyes are released into a river, they either stay in the liquid phase (Garrison and Hill, 1972; Maguire and Tkacz, 1991) or are adsorbed to the ground sediment and bioconcentrated there. The anoxic conditions of the sediment facilitate the azo bond to be reduced resulting in the discharge of aromatic amines (Weber and Adams, 1995).

Attempts to lower the mutagenicity in industrial wastewaters containing azo dyes and their decomposition products are based on the combination of anaerobic and aerobic water treatment (Cruz and Buitron, 2001; Ekici et al., 2001), ozonation (Gahr et al., 1994) or the usage of non-mutagenic dyes (Freeman et al., 1990). (G. de Aragão Umbuzeiro et al., 2003)

3. Polycyclic aromatic compounds under consideration

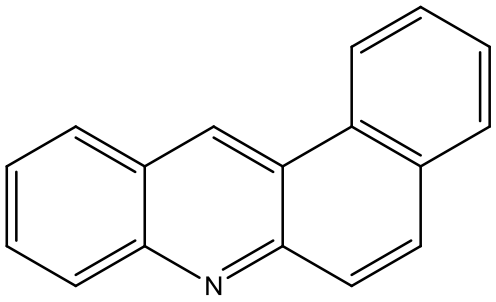
- **Acridine**

Figure 2: Structure of Acridine



- **Benzo[a]acridine**

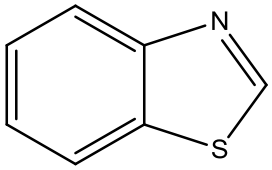
Figure 3: Structure of Benzo[a]acridine



Benzo[c]acridine, having a similar structure to benzo[a]acridine, has been proven to cause skin tumours in mice treated topically and bladder tumours in rats after local paraffin wax pellet implantation (IARC, 1998).

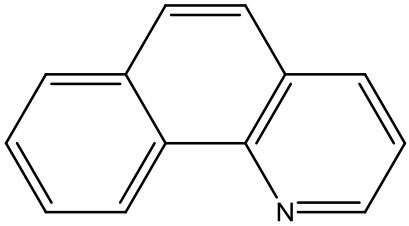
- **Benzothiazole**

Figure 4: Structure of Benzothiazole



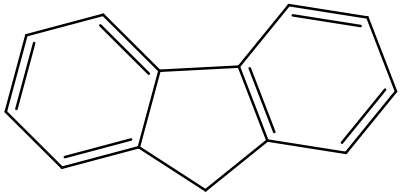
- **Benzo[h]quinoline**

Figure 5: Structure of Benzo[h]quinoline



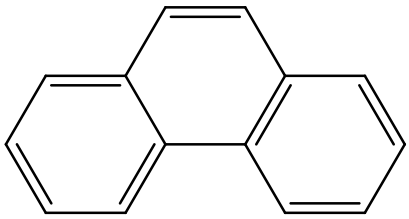
- **Fluorene**

Figure 6: Structure of Fluorene



- **Phenanthrene**

Figure 7: Structure of Phenanthrene



The results of studies on phenanthrene are depicted in tables 2.1- 2.4 showing a positive result of tumour initiation.

Dermal application to mice

A group of 20 male C3H/HeJ mice, 6-8 weeks of age, received twice-weekly skin applications of 50 µL of a 0.1% toluene solution of phenanthrene (>99% pure by HPLC; 50 µg per treatment) for 104 weeks. A control group of 50 male mice was treated with toluene alone. Lesions (≥ 1 mm^3) that persisted for at least 1 week were diagnosed as papillomas. After 104 weeks, one benign skin tumour was observed in the 12 surviving experimental mice and no benign or malignant skin tumours in the 39 surviving control mice. Gross examination of internal organs indicated no tumours in either the experimental or control groups. (Warshawsky et al., 1993)

Intrapulmonary administration to rats

Groups of 35 inbred female Osborne-Mendel rats, 3 months of age and weighing on average 256 g, received a single pulmonary implantation of 1, 3 or 10 mg phenanthrene (purity 99.9%) in a mixture of beeswax and tricaprylin. The animals were monitored for 132-135 weeks. A single lung carcinoma was found in the high- dose phenanthrene-treated group. No lung carcinomas developed in solvent-treated control rats. (Wenzel-Hartung et al., 1990)

Table 2.1.: Carcinogenicity studies of dermal application of phenanthrene in experimental animals

Chemical, species and strain	Sex	No./group at start	Purity (vehicle)	Dose and duration of exposure	Duration of study	Incidence of tumours	Result[a]	Comments	Reference
Phenanthrene									
Mouse, NS	NS	100	NS (90% benzene)	NS	9 mo	0/100 skin	–	Limited reporting; no control	Kennaway (1924a,b)
Mouse, white	NS	100	'Pure' (5% croton oil)	3 drops of a 3% solution, 1×wk	1 year	1/6 (17%)	±	Low survival; no statistics	Graffi *et al.* (1953)
Mouse, C3H/HeJ	M	20, 50 controls	>99% (toluene)	0, 50 µg in 50 µL, 2×/wk, 104 wk	104 wk	1/12 (8%) benign skin T vs 0/39 benign or malignant skin T solvent controls	–	No statistics	Warshawsky *et al.* (1993)

A, adenoma; C, carcinoma; DMF, *N,N*- dimethylformamide; DMSO, dimethylsulfoxide; E, epithelioma; F, female; K, keratocanthoma; M, male; mo, month; NH_4OH, ammonium hydroxide; NS, not specified; P, papilloma; PAH, polycyclic aromatic hydrocarbon; S, sarcoma; SCC, squamous-cell carcinoma; SGA, sebaceous gland adenoma; T, tumour; TBA, tumour-bearing animal; TPA, 12-*O*-tetradecanoyl-13-acetate; vs, versus; wk, week

[a] –, negative; +, positive; ±, equivocal

Table 2.2.: Dermal initiation-promotion studies of phenanthrene in experimental animals

Chemical, species and strain	Sex	No./group at start	Purity (vehicle)	Dose and duration of exposure	Duration of study	Incidence of tumours	Result[a]	Comments	Reference
Phenanthrene									
Mouse, 'S'	NS	20	NS (acetone)	0, 54 mg, 10×, 3×/wk, followed by (0.17%) croton oil, 300 µL, 1×/wk, 16 wk; 300 µL (0.085%) 1×/wk, 2 wk	24 wk	5/20 (25%) skin P (12 T) vs 4/19 (21%) skin P (4 T) croton oil controls	±		Salaman & Roe (1956)
Mouse, 'stock albino'	M, F	10	'High purity' (acetone)	0, 300 µg on days 0, 2, 6, 8, followed by 250 µl (0.1%) croton oil, 1×/wk, 20 wk	24 wk	4/19 (21%) skin P vs 2/20 (10%) skin P croton oil controls	±		Roe (1962)
Mouse, CD-1	F	30	Purified (acetone)	0, 1.78 mg, 1×, followed by 5 µmol TPA, 2×/wk, 34 wk	35 wk	12/30 (40%) skin P vs 1/30 (3%) skin P TPA controls	+	No histology; no statistics	Scribner (1973)
Mouse, CD-1	F	30	>98% (acetone: ammonium hydroxide (1000:1))	0, 1.78 mg, 1×, followed by 16 nmol TPA, 2×/wk, 35 wk	36 wk	4/30 (13%) skin P vs 2/30 (7%) skin P TPA controls	±	No histology	Wood *et al.* (1979)
Mouse, Swiss albino Ha/ICR	F	20	>99.5% (acetone)	0, 100 µg in 100 µL, 10× every other day, followed by 2.5 µg TPA, 3×/wk, 20 wk	24 wk	0/20 skin P vs 0/20 skin P TPA controls	–	No histology	LaVoie *et al.* (1981)

C, carcinoma; DMSO, dimethylsulfoxide; F, female; M, male; mo, month; NS, not specified; P, papilloma; PAH, polycyclic aromatic hydrocarbon; SCC, squamous-cell carcinoma; SE, standard error; SGA, sebaceous gland adenoma; T, tumour; THF, tetrahydrofuran, TPA, 12-*O*-tetradecanoylphorbol-19-acetate; UV, ultraviolet; vs, versus; wk, week
[a]–, negative; +, positive; ±, equivocal

Table 2.3.: Carcinogenicity studies of subcutaneous administration of phenanthrene in experimental animals

Chemical, species and strain	Sex	No./group at start	Purity (vehicle)	Dose and duration of exposure	Duration of study	Incidence of tumours	Result[a]	Comments	Reference
Phenanthrene									
Mouse, C57BL	M, F	40–50	NS (tricaprylin)	5 mg, 1×	22–28 mo	0/27 surviving >4 mo	–	No control	Steiner (1955)
Mouse, 'stock albino'	M, F	10	NS (3% aqueous gelatine)	0, 300 µg on days 0, 2, 6, 8, followed by 250 µL croton oil, 1×/wk, 20 wk	24 wk	3/17 (18%) skin P vs 2/20 (10%) skin P croton oil control	–		Roe (1962)

A, adenoma; AdC, adenocarcinoma; av., average; C, carcinoma; DMSO, dimethylsulfoxide; F, female; M, male; mo, months; NS, not specified; P, papilloma; PAH, polycyclic aromatic hydrocarbon; PND, postnatal day; S, sarcoma; SCC, squamous-cell carcinoma; T, tumour; TBA, tumour-bearing animal; vs, versus; wk, week
[a]–, negative; +, positive; ±, equivocal

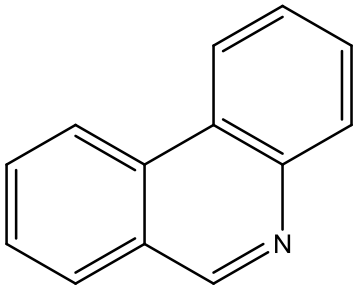
Table 2.4.: Carcinogenicity study of oral administration of phenanthrene in experimental animals

Chemical, species and strain	Sex	No./group at start	Purity (vehicle)	Dose and duration of exposure	Duration of study	Incidence of tumours	Result[a]	Comments	Reference
Phenanthrene									
Rat, Sprague-Dawley	F	10	NS (sesame oil)	200 mg, 1×	60 days	0/10 vs 8/164 (5%) mammary gland T non-concurrent controls after 310 days	–	Small numbers; no statistics	Huggins & Yang (1962)

A, adenoma; AdC, adenocarcinoma; C, carcinoma; F, female; H, hepatoma; M, male; NS, not specified; P, papilloma; PAH, polycyclic aromatic hydrocarbon; S, sarcoma; SCC, squamous-cell carcinoma; SE, standard error; T, tumour, TBA, tumour-bearing animal; vs, versus; wk, week
[a]–, negative; +, positive; ±, equivocal

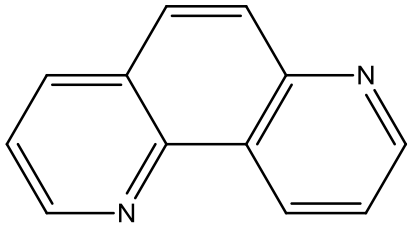
- **Phenanthridine**

Figure 8: Structure of Phenanthridine



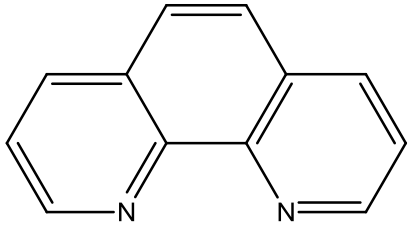
- **1,7-Phenanthroline**

Figure 9: Structure of 1,7-Phenanthroline



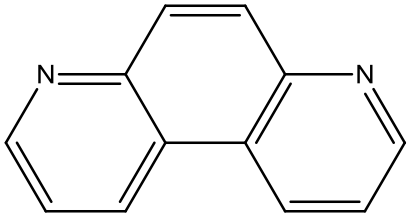
- **1,10-Phenanthroline**

Figure 10: Structure of 1,10-Phenanthroline



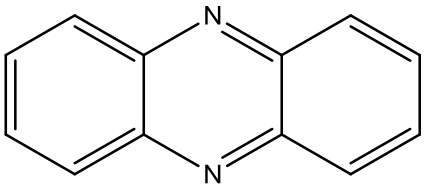
- **4,7-Phenanthroline**

Figure 11: Structure of 4,7-Phenanthroline



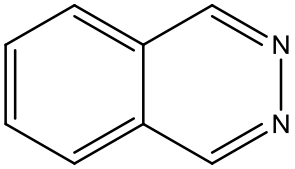
- **Phenazine**

Figure 12: Structure of Phenazine



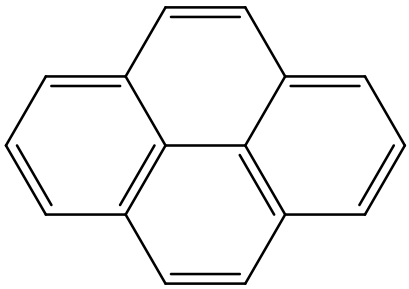
- **Phthalazine**

Figure 13: Structure of Phthalazine



- **Pyrene**

Figure 14: Structure of Pyrene



Dermal application to mice

In a co-carcinogenicity study in male C3H/HeJ mice, a solution of 0.1% pyrene applied to the skin simultaneously with 0.001% benzo[a]pyrene did not induce skin tumours; one mouse (8%) treated with pyrene alone developed a skin tumour; mice treated with benzo[a]pyrene alone and vehicle (toluene) controls did not develop skin tumours. (Warshawsky et al., 1993)

Table 3 summarizes the study results of the dermal application of several PAHs and their mixtures to mice. (Warshawsky et al., 1993)

Table 4 shows the outcomes of carcinogenicity studies of intraperitoneal administration of pyrene in experimental animals.

Table 3: Incidence of benign and malignant skin tumours in mice treated with some PAHs, including pyrene, and mixtures of PAHs

Compound	Malignant skin tumours	Benign and malignant skin tumours combined
Toluene	0/20	0/39
Benzo[*a*]pyrene	19/20 (95%)	0/14
Benzo[*a*]pyrene + chrysene	13/16 (81%)	3/13 (23%)
Benzo[a]pyrene + phenanthrene + pyrene	18/19 (95%)	19/19 (100%)
Benzo[*a*]pyrene + anthracene	1/13 (8%)	1/13 (8%)
Benzo[*a*]pyrene + fluoranthene		1/12 (8%)
Benzo[*a*]pyrene + phenanthrene		1/17 (6%)
Benzo[*a*]pyrene + pyrene		0/13
Benzo[*a*]pyrene + anthracene, chrysene, fluoranthene, phenanthrene and pyrene		8/17* (47%)
Anthracene		0/14
Chrysene		1/15 (7%)
Fluoranthene		0/15
Phenanthrene		1/12 (8%)
Pyrene		1/13 (8%)
Anthracene, chrysene, fluoranthene, phenanthrene and pyrene		3/13 (23%)

From Warshawsky *et al.* (1993)
PAH, polycyclic aromatic hydrocarbon
*Significantly different from benzo[*a*]pyrene alone

Table 4: Carcinogenicity studies of intraperitoneal administration of pyrene in experimental animals

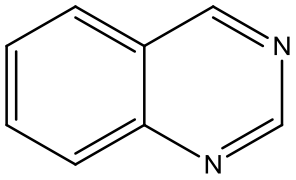
Chemical, species and strain	Sex	No./group at start	Purity (vehicle)	Dose and duration of exposure	Duration of study	Incidence of tumours	Result[a]	Comments	Reference
Pyrene									
Mouse, CD-1	M, F	90 or 100	>99% (DMSO)	0, 700 nmol (total dose; given as 1/7, 2/7, 4/7 of total dose on PND 1, 8, 15)	52 wk	**Lung A/AdC** M: 9%, 8%; F: 6%, 10% **Liver A/C** M: 11%, 12%; F: 0%, 0% **Lymphoma** F: 0%, 10%	–		Wislocki *et al.* (1986)
Mouse, CD-1	M, F	90 or 100	>99%, (DMSO)	0, 200, 2800 nmol (total dose; given as 1/7, 2/7, 4/7 on PND 1, 8, 15)	52 wk	**Lung A/AdC** M: 4%, 3%, 7%; F: 0%, 3%, 6% **Liver A/C** M: 7%, 0%, 21%; F: 0%, 0%, 0% **Lymphoma** M: 4%, 0%, 0%; F: 3%, 3%, 6%	–		Wislocki *et al.* (1986)
Mouse, Swiss-Webster BLU:Ha (ICR)	M, F	NS	>98% (DMSO)	0, 0.43, 9.65 µmol (total dose; given as 1/7, 2/7, 4/7 on PND 1, 8, 15)	26 wk	**Lung A/AdC (T/mouse)** M: 13/91 (14%; 0.15 ± 0.04), 4/23 (17%; 0.17 ± 0.08), 2/27 (7%; 0.07 ± 0.05) F: 7/101 (7%; 0.08 ± 0.03), 1/28 (4%; 0.04 ± 0.04), 3/26 (12%; 0.12 ± 0.06)	M, –; F, ±		Busby *et al.* (1989)
Mouse, strain A/J	M	20	99.7% (tricaprylin)	0, 10, 50, 100, 200 mg/kg bw, 1 ×	8 months	No induction of lung T at any of the doses administered	–		Ross *et al.* (1995)

A, adenoma; AdC, adenocarcinoma; C, carcinoma; DMSO, dimethylsulfoxide; F, female; H, hepatoma; M, male; NS, not specified; P, papilloma; PAH, polycyclic aromatic hydrocarbon; PND, postnatal day; S, sarcoma; SCC, squamous-cell carcinoma; SEM, standard error of the mean; T, tumour; TBA, tumour-bearing animal; vs versus wk, week

[a]–, negative; +, positive; ±, equivocal

- **Quinazoline**

Figure 15: Structure of Quinazoline



- **Quinoline**

Figure 16: Structure of Quinoline

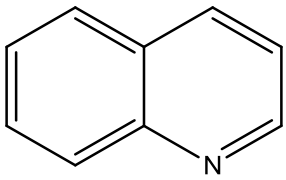


Table 5 lists the carcinogenic compounds that were released in the United States in 2001 (Ohe et al., 2004). Among them are also polycyclic aromatic hydrocarbons highlighted in the table.

Table 5: Carcinogenic compounds released in the US in 2001

Compound	Mutagenicity/ clastogenicity[a,b]	Carcinogenicity[c]	Total water releases[d] ($\times 10^3$ kg)	Total air emissions[e] ($\times 10^6$ kg)
Lead compounds[f]	##	2B	164.3	569.0
Formaldehyde	+++/##	2A	152.5	4,800.1
Nickel compounds[g]	++/##	1	111.1	455.7
Chromium compounds[h]	+++/##	1	80.9	304.4
Acetaldehyde	+++/##	2B	71.4	5,397.6
Arsenic compounds[i]	+/##	1	64	–
1,4-Dioxane	+[j]/#	2B	36.9	–
Cobalt compounds[k]	++/##	2B	21.8	–
N,N-Dimethylformamide	++/#	3	17	242.6
Benzene	+++/##	1	9.6	2,673.8
Chloroform	+/##	2B	8.6	647.6
Catechol	++/#	2B	7.8	–
Polycyclic aromatic compounds[l]			7.4	519.9
Benz(a)anthracene	+++/##	2A		
Benzo(a)pyrene	+++/##	2A		
Benzo(b)fluoranthene	+/#	2B		
Dibenzo(a,h)anthracene	++/##	2A		
Indeno(1,2,3-cd)pyrene	+	2B		
Dibenz(a,h)acridine	+	2B		
Beryllium compounds[m]	++/#	1	4.6	–
Ethylbenzene	+[n]	2B	4	2,969.9
Epichlorohydrin	++/##	2A	3.5	–
Diaminotoluene (mixed isomers)	–	2B[o]	2.7	–
Dichloromethane	++/##	2B	2.2	9,778.4
Ethylene oxide	+++/##	1	2.1	–
Styrene	+++/##	2B	1.4	21,077.0
Cadmium compounds[p]	++/##	1	1.1	–
Creosote	++	2A	1.1	–
Trichloroetylene	+	2A	–	3,741.9
Vinyl acetate	#	2B	–	1,303.9
Tetrachloroetylene	–	2A	–	1,213.2
1,3-Butadiene	##	2A	–	973.0

Table 5 continued

Compound	Mutagenicity/ clastogenicity[a,b]	Carcinogenicity[c]	Total water releases[d] ($\times 10^3$ kg)	Total air emissions[e] ($\times 10^6$ kg)
Acrylnitrile	+++	2A	–	424.5
Chloroprene	+++/##	3	–	386.5
Vinyl chloride	+++/##	1	–	332.1
Total			776.0	57811.1

[a] Based on data from references [8–10].

[b] –, compounds for which there is no evidence of mutagenicity or clastogenicity; +, mutagenic in bacterial and/or fungal/yeast cells in vitro; ++, also mutagenic in plants or animal cells in vitro; +++, also mutagenic in the *Drosophila melanogaster* somatic mutation and recombination test, and/or sex-linked recessive lethal test, and/or transgenic rodent assays, and/or rodent dominant lethal test. For cytogenetic endpoints, # refers to substances are clastogenic in in vitro or in vivo assays, ## refers to substances that are clastogenic both in vitro and in vivo. *Note*: In some instances conflicting results have been reported in the literature.

[c] IARC classification system: 1—carcinogenic to humans, 2A—probably carcinogenic to humans, 2B—possibly carcinogenic to humans, 3—inadequate or limited evidence of carcinogenicity in experimental animals. IARC monographs on the evaluation of carcinogenic risks to humans, volumess 11, 15, 16, 23, 32, 47, 49, 52, 54, 58, 60, 62, 63, 71, 73, 77, and supplements 6 and 7. International Agency for Research on Cancer, Lyon, France.

[d] >1000 kg only.

[e] >3 × 1000 kg only.

[f] Various compounds.

[g] Nickel(II) salts (e.g., $NiCl_2$) and insoluble crystalline nickel (e.g., Ni_3S_2).

[h] Hexavalent chromium compounds only (e.g., $K_2Cr_2O_7$, K_2CrO_4).

[i] Both the +3 and +5 oxidation states are clastogenic in vitro.

[j] Rodent dominant lethal assay only.

[k] Cobalt (II) salts only (e.g., $CoCl_2$).

[l] The TRI lists PACs (polycyclic aromatic compounds) as a category of 19 individual compounds. A list of compounds included is available at http://www.epa.gov/tri/chemical/chemlist2001.pdf.

[m] Primarily beryllium (II) compounds (e.g., $BeSO_4$).

[n] Animal cells only.

[o] Only 2,4-diaminotoluene evaluated.

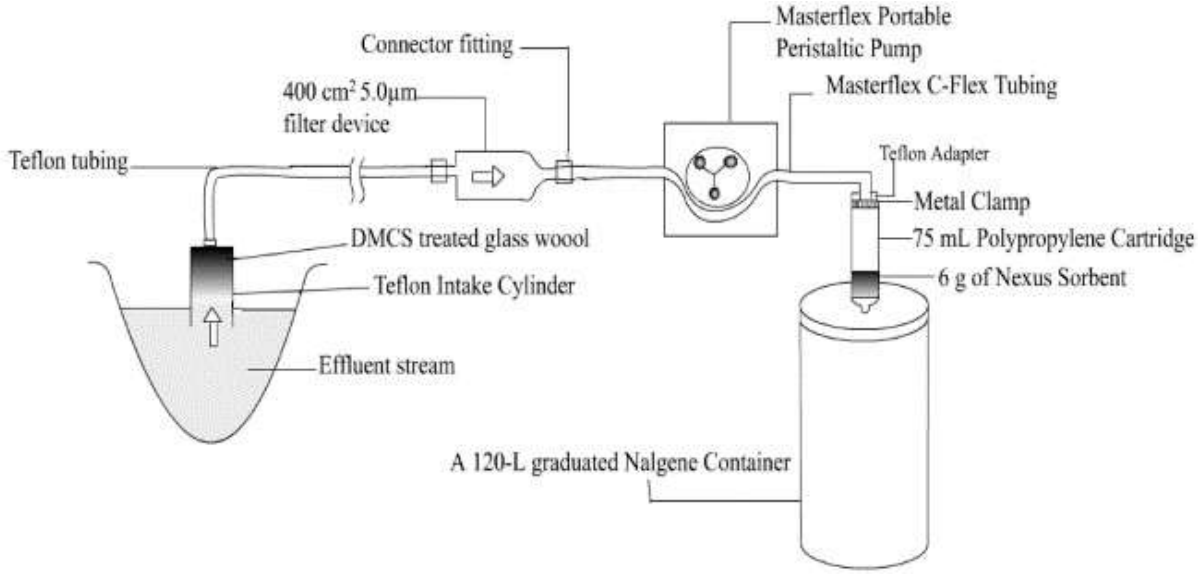
[p] Cadmium (II) salts only (e.g., $CdCl_2$).

4. Sample concentration techniques

Although the quantities of mutagens in the river water are often even below the detection limit of analytical instruments they have to be taken into account, since the bioaccumulation index of the majority of mutagens is quite high. To achieve trustable results concerning the concentration of mutagens special pre-concentration techniques have to be used. (S. Britvic et al., 2010)

On-site concentration of the examined compounds from a water sample simplifies the transport of the analytes to the laboratory (Kira et al., 1998b). Figure 17 (L.I. Osemwengie, S. Steinberg, 2001) depicts an apparatus suitable for the on-site concentration.

Figure 17: On-site Solid-Phase Extraction Assembly



4.1. The Blue Cotton technique

The repressive impact of hemin and other porphyrins on the activity of specific mutagens (Arimoto et al., 1980 a,b), namely the ones with planar structures including three or more fused rings has already been known before the examination of phthalocyanine. Since phthalocyanine has an assimilable chemical structure to porphyrin, copper phthalocyanine derivatives, blue pigments frequently utilized as dyes, were supposed to also have an affinity to mutagens with planar structures. (H. Hayatsu et al., 1982)

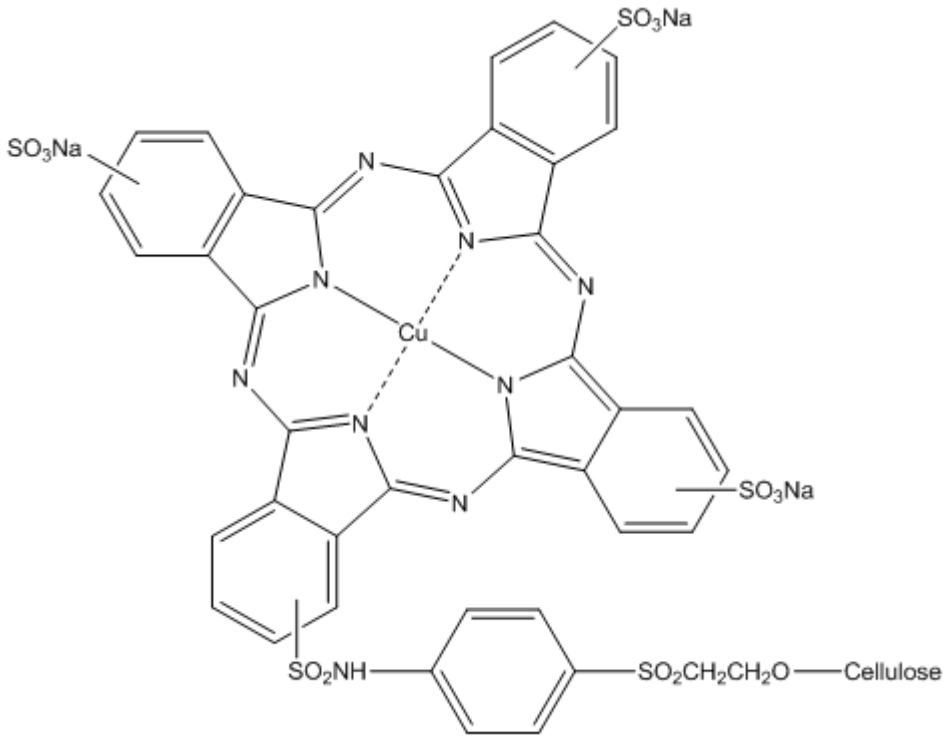
Mutagenicity tests of chalks used for teaching revealed positive results in the Salmonella test only for blue chalks. The blue pigment was identified as phthalocyanine and the attempt to make use of it in pre-concentration appeared. As material for carrying this blue pigment cotton seemed to be suitable due to its swelling in water, which leads to an efficient contact with mutagens in solution. (Hayatsu, 1992)

So it was found out that cotton carrying covalently bound trisulfo-copper-phthalocyanine is able to adsorb mutagens with polycyclic planar structures. This modified cotton can be utilized for the concentration of mutagens from river water later desorbing them with organic solvents.

For the preparation of a Blue Cotton absorbent, cotton has to be treated with C.I. Reactive Blue 21 in water containing sodium carbonate and sodium sulphate. For the coupling a temperature of 30°C has to be kept for 35 minutes.

Afterwards the mixture has to be heated to 70°C for 60 minutes. The following structure, seen in Figure 18, is the result of this procedure.

Figure 18: Structure of cotton-bound pigment



For the usage of Blue Cotton the mutagens can be dissolved in saline. The adsorption to the Blue Cotton is induced by shaking the mutagen solution with the Blue Cotton added.

When determining mutagens from a river the Blue Cotton is hung in the flowing water to serve as an adsorbent. Usually it is removed from the river after 24 hours. Then the mutagens are eluted with ammonical methanol. Molecules consisting of two or less aromatic rings are not adsorbed much to Blue Cotton. The adsorption to Blue Cotton depends on the pigment bound to the cellulose rather than on the pH.

Histidine and oleic acid, which constrict the Ames test (Hayatsu et al., 1981), are not adsorbed to Blue Cotton. This is why the action of frameshift mutagens pre-concentrated using Blue Cotton can be tested by Ames test on strain TA98 or TA100 in the presence of S9.

The Blue Cotton technique is easy in handling and suitable for the adsorbtion of numerous mutagens. Contaminating material does not have to be eliminated from the Blue Cotton

before the usage for adsorption. Therefore it has many benefits compared to other adsorption methods. (H. Hayatsu et al., 1982)

Washing procedure of Blue Rayon

Blue rayon can be recycled when a standardized and optimized procedure of washing is performed first.

Therefore, the Blue Rayon has to be stirred four times in ultra-pure water for 5 minutes disposing the water carefully afterwards.

Then the Blue Rayon is shaken repeatedly in a methanol/ammonia (50:1 v/v) solution for 1hour. The Blue Rayon is kept in the solution overnight. Afterwards it is put in methanol for 1hour while agitating it from time to time. Part of the methanol is disposed and the remaining liquid is reduced to 2-3 mL with a rotary evaporator. The left methanol is evaporated to dryness in a small vial under nitrogen flow. Dimethylsulfoxide is added. To prove the completeness of the elimination of mutagenic contaminants a *Salmonella* mutagenicity assay has to be conducted. (F. Kummrow et al, 2003)

Consideration of river flow rate intensity

Besides the concentration of polycyclic aromatic hydrocarbons an aspect that has to be taken into account when using the Blue Rayon hanging technique is the influence of the flow rate of the examined river on the extent of adsorption of mutagens to the Blue Rayon (Kira et al., 1996). The water flow rate can be assessed using the plaster ball method (Komatsu and Kawai, 1992), which was already utilized in a study of Kira et al. (Kira et al, 1998a) in which a modified Blue Rayon technique was employed to determine benzo[a]pyrene in the sea.

Hanging a gypsum ball into the analyzed water, it is solubilised partly, thereby losing weight. The observed loss in mass is directly proportional to the flow rate of the water. For standardizing the plaster ball a ping-pong ball is suitable as a cast. Initially the balls have to be submerged in distilled water for an hour, afterwards gently drying the ball with a paper towel.

The initial weight (W_0) of each ball is recorded. After putting the balls into plastic nets one ball is positioned 5 cm above and the other one 5 cm below 1 g of Blue Rayon (Kira et al., 1998a). The nets were fixed on a Styrofoam float 30-50 cm under the water surface. After 24

hours the balls were dried again with a paper towel and their average final weight loss was determined according to equation 1. The weight loss represents the flow rate of the water surrounding the Blue Rayon.

Equation 1: weight loss

$$\Delta W = W_0 - W$$

To conclude, the faster the water movement, the greater the adsorbtion of polycyclic aromatic hydrocarbons to the Blue rayon, when the concentration of PAHs in the water is constant.

Equation 2: Proportionality between rate of water movement and adsorption of PAHs

$$\frac{v_H}{v_L} \propto \frac{A_H}{A_L}$$

v_H ….. velocity of fast water movement
v_L ….. velocity of slow water movement
A_H ….. adsorbed larger amount of PAH
A_L ….. adsorbed smaller amount of PAH

The more rapid the water flow, the greater the weight loss of the gypsum ball.

Equation 3: Proportionality of rate of water flow and the weight loss of the plaster ball

$$\frac{v_H}{v_L} \propto \frac{\Delta W_H}{\Delta W_L}$$

ΔW_H ….. weight loss observed at higher flow rate
ΔW_L ….. weight loss observed at lower flow rate

The procedure was standardized in the following way. Using the modified Blue Rayon hanging technique described above the time-weighted average concentration (TWA) of benzo[a]pyrene [TWA-BaP] was determined (Kira et al., 1997).

The concentration of mutagens adsorbed on the Blue Rayon [BR-BaP] was correlated with the time-weighted average concentration via equation 4.

Equation 4: correlation of concentration of adsorbed mutagens to time-weighted average concentration

$$[\mathrm{TWA-BaP}] = \mathrm{K} \cdot \frac{[\mathrm{BR-BaP}]}{\Delta \mathrm{W}^{\mathrm{m}}}$$

K, m ….. constants

[TWA-BaP] ….. time-weighted average concentration of benzo[a]pyrene

[BR-BaP] ….. concentration of benzo[a]pyrene adsorbed to blue rayon when using the original blue rayon technique without modifications

The best correlation coefficient (0.749, n = 29) was given when m and K were about 1 and 6.5 respectively.

Taking this into account it is possible to convert the BaP concentration from ng $g(BR)^{-1}$ to ng L^{-1} of TWA via equation 5.

Equation 5: conversion of BaP concentration into time-weighted average concentration

$$[\mathrm{TWA-BaP}] = 6.5 \cdot \frac{[\mathrm{BR-BaP}]}{\Delta \mathrm{W}}$$

The calculated time-weighted average concentration of BaP considered the BaP in the liquid phase as well as the BaP adsorbed to particles. (S. Kira et al., 1998a)

4.2. Adsorption on Amberlite XAD resins

XAD resins can adsorb lots of mutagenic compounds including polycyclic aromatic hydrocarbons, arylamines, nitro-compounds, quinolines and anthraquinones.

In the XAD resin attempt the water samples are first extracted at neutral pH consequently extracting them under acidic pH. XAD4 resin, a copolymer of styrene divinyl benzene, is used in a concentration of 1 mL L^{-1} surface water and 0.5 mL L^{-1} wastewater at a flow rate of 100 mL min^{-1}. The extract from the neutral pH extraction is eluted with 1 mL methanol and 4 mL methylene chloride per mL of resin. The acidic extract is eluted replacing the methylene chloride by 4 mL ethyl acetate. Both elutions are conducted at a rate of 2 mL min^{-1}. Afterwards the eluates are evaporated until 2-3 mL remain, which are evaporated to dryness under nitrogen stream. The mutagens are resuspended in dimethylsulfoxide and can be used for the qualitative and quantitative analysis.

While at natural pH mainly non-polar compounds are adsorbed to XAD4 resin, disinfection by-products, which mostly show higher polarity, are preferentially adsorbed to the XAD4 resin at an acidic pH. (F. Krummrow et al., 2003)

4.3. The blue-chitin column method

The blue-chitin column method is perfectly suitable for the concentration of polycyclic compounds from river water.

Like in the Blue rayon technique the blue pigment cpt is used. Therefore, it is linked to chitin (=poly-N-acetlyglucosamine).

To optimize the results of the blue-chitin column method the rate of sample-water should be maximized. It has been proved that the speed can be increased up to 20 mL min^{-1}. Studies have shown that adsorption is more efficient using several smaller volumes for the passage through numerous columns than using just one column and a higher water sample volume at once.

Most trustable results are achieved when passing a 1-liter sample through a single column or applying two serially connected columns for a 5-liter sample.

4.4. Comparison of the Blue-Rayon hanging, XAD-resin column and the blue-chitin column techniques

Its simplicity and selectivity for polycyclic aromatic compounds turns the Blue Rayon technique into a good method for the qualitative determination of river water mutagenicity. The blue (cpt)-chitin column concentration is suitable for quantitative analysis of polycyclic compounds from river water.

While the non-polar copolymers of styrene-divinylbenzene, polystyrene-based XAD-resins, adsorb nearly all organic compounds, the Blue Rayon technique is selective for polycyclic compounds: e.g., mutagenic heterocyclic amines, polycyclic hydrocarbons, psoralens, and aflatoxins. Thus, mutagens like dialkylnitrosamines can be collected effectively by using XAD resins rather than cpt adsorbents.

Due to the fact that organic compounds like long-chain fatty acids are attached to XAD resins and block the *Salmonella* assays of mutagens it might be reasonable why in XAD resin concentrates lower mutagenicity has been observed. Another possible reason for that

might be that the affinity of polycyclic mutagens to XAD-resins is lower than to cpt-adsorbents.

When the XAD-2 concentration and the blue-chitin technique were compared it was found out that, the blue-chitin column method showed a significantly higher number of colonies in the *Salmonella* assay. In conclusion, the blue-chitin column method is more efficient than using XAD-2 resins. Table 6 summarizes the unique features of the blue-chitin column method and the blue- rayon hanging technique.

Table 6: Features of blue-chitin and blue-rayon techniques for monitoring river water mutagens

	Blue-chitin column	**Blue-rayon hanging**
Quantitativeness	quantitative	semi-quantitative
Time for work	3 – 6 h work-up	24 h hanging; 2 – 3 h work-up
Site-visit number	1	2
Other features	spot sampling; experiments repeatable with stored water samples	1-day exposure sampling; suitable for collecting large amounts of target substances; water transport to laboratory unnecessary

(H.Sakamoto et al., 1996)

4.5. Liquid-liquid extraction

Liquid-liquid extraction with organic solvents is a frequently utilized method to receive quantitative information on mutagenic compounds. Compared to XAD resin concentrates the method was less effective (Rehana et al. 1995, 1996; Takamura-Enya et al., 2002; White et al., 1999). (Ohe et al., 2004)

5. Qualitative and Quantitative Analysis of Pollutants

5.1. High-Performance Liquid Chromatography

High-Performance Liquid Chromatography has been frequently used in the analysis of BaP. Therefore, BaP in the sample water has to be pre-concentrated. Summarizing this process (Kira et al., 1995) shortly, a Nova-Pak C18 column (ϕ 3.9 × 150 mm, 4 μm, Waters, Millipore, Millford, MA, USA) is used: mobile phase, acetonitrile-water (65/35); flow rate, 1.0 mL/min; temperature, 40°C. A fluorescent spectrophotometer with excitation and emission wavelengths set to 365 and 406 nm respectively is utilized for the quantitative analysis. Peak areas are calculated by an integrator.

To prove the reliability of the results of the method, the procedure is conducted with a known amount of BaP, for example, 10 ng = 10 μL of 1 ng/μL methanol solution, can be spiked into 1 L of distilled water in a PET bottle. A former study of Kira et al. proceeded in the described way resulted in a recovery rate of 39.8 ± 1.6% (mean ± SD, n = 6) for the BaP with 7% of CV for the extraction procedure. (Kira et al., 1998b)

6. Measurement of the BaPMO activity

6.1. The MFO inducting-test

As biomarker and bioindicator of exposure to organic pollutants such as PAHs, PCBs, pesticides and PCDD/F, which are caused by human activities an induction of cytochrome P450-dependent monooxygenase system in fish liver can be utilized (Buhler and Wang-Buhler, 1998; Parrott et al., 2000).

For the MFO inducting experiment a one year old carp is kept in a flow of dechlorinated, well aerated tap water for three month. Further, native fish from the river to be analysed are caught. The liver of all fish is later separated and used for measurement of BaPMO activity.

Therefore, the mutagens contained in 1, 3 and 6 L of river water are pre-concentrated by the Blue Cotton technique. The extract solution is evaporated to dryness and resuspended in 4 mL of corn oil. Then the extract is intraperitoneally injected in the experimental carp.

As a positive control a carp intraperitoneally treated with 3-methylcholanthrene is used. For the negative control only corn oil is injected into another carp. No controls were applied for the native fish species.

After three days, the 3-hydroxy-benzo[a]pyrene concentration in the liver of the carps is measured. The method of Nebert and Gelboin (Nebert and Gelboin, 1968) can be applied to determine the BaPMO activity in liver post-mitochondrial fractions (S9).

The same is done with the native fish species. (Britvić et al., 2010)

7. Determination of mutagenicity

7.1. Ames mutagenicity test

The Ames *Salmonella*/microsome mutagenicity assay is based on bacterial reverse mutations and is widely used for the evaluation of numerous chemicals, having an impact on the DNA, thereby inducing gene mutations. (K. Mortelmans et al., 2000)

Performing Ames test strains of the bacterium *Salmonella* typhimurium including mutated genes involved in histidine synthesis are used. Since this mutants need histidine for their growth, which the original not mutated strains did not need they are so called auxotrophic mutants. When these strains are induced to mutate back to the state in which histidine is no longer a precondition for their growth they can also invade a medium free from histidine. (B.N. Ames, 1972)

To imitate the effect of metabolism a rat liver extract can be added. This might be necessary for accurate results since some compounds, like benzo[a]pyrene, are not mutagenic themselves but their metabolic products are. (B.N. Ames, 1973)

The bacterial strains and the sample are dispersed on an agar plate carrying some histidine in the growth medium, so that the initial bacteria can grow and mutate on the medium.

When all histidine is consumed, only the mutated colonies, which are able to produce histidine on their own, survive. After 48 hours of incubation the revertant colonies can be counted and serve as direct indicator of mutagenicity. (J. McCann et al., 1975)

A value of MR is calculated. Therefore, the number of revertant colonies in the sample plates is divided by the number of spontaneous revertants. A MR higher than two indicates the mutagenicity of the substance, while a value below two can be interpreted as the absence of mutagenicity. (Britvić et al., 2010)

Substances categorized as mutagenic via the Ames test are very likely carcinogens. 90 percent of known carcinogens show a positive result in Ames test and only few non-carcinogens lead to a positive result in Ames test. This fact turns the Ames test into a very reliable technique for the estimation of the carcinogenicity of chemicals. (J. McCann et al., 1975)

7.2. The SOS Chromotest

The SOS chromotest is a quantitative bacterial colorimetric method for the evaluation of DNA-damaging agents, so called genotoxins. *Escherichia coli* react to genotoxins in several ways, known as SOS responses (Radman, 1975; Witkin, 1976; Devoret, 1981; Little and Mount, 1982; Walker, 1984) and din genes (Kenyon and Walker, 1980). For the test an operon fusion is proceeded placing lacZ, which is the structural gene for *β*-galactosidase, under the impact of the sfiA gene (Huisman and d'Ari, 1981), a SOS function capable of the cell-division blocking (Quillardet et al., 1982). The utilized *E. coli* PQ37 sample strain (Quillardet et al., 1982) contains a sfiA-lacZ fusion and a lacks the usual lac region. Consequently the *β*-galactosidase activity relies on the SOS response, the expression of sfiA, which is only the case in presence of a genotoxin. Due to a mutation the strain is not capable of excision repair and therefore, the response to certain DNA-damaging agents is altered. Further, another mutation of the strain causes a lack of lipopolysaccharide, thereby leading to a more intense diffusion of chemicals into the cell. The tester strains have to be incubated with increasing concentrations of the examined substance. When the protein synthesis has taken place, the *β*-galactosidase activity is measured and corresponds to the amount of genotoxins.

The release and induction of *β*-galactosidase by the tester strain is observed on indicator plates based on the substrate 5-bromo-4-chloro-3-indolyl-*β-D*-galactosidase. In case the substrate is hydrolyzed by *β*-galactosidase a coloured dye release is observed. The whole testing procedure is conducted in agar as a SOS spot test on a plate. The results of the method are gained in few hours. (P. Quillardet and M. Hofnung, 1984)

7.3. The umu-test

The umu-test uses the facility of genotoxins to cause the expression of the umu operon in *E. coli*, which is capable of chemical and radiation mutagenesis. A plasmid including the fused gene umuC - lacZ is inserted into *Salmonella* typhimurium TA1535/pSK1002. This strain makes it possible to monitor the expression of the umu operon by measuring the *β*-galactosidase activity originating from the fusion gene. (Y. Oda et al., 1985)

8. Determination of genotoxicity

8.1. The *Allium cepa* test

The assessment of the danger of DNA-damage and toxicity of mixtures of contaminants has been attempted in several ways, making use of microorganisms, plant cells and mammalian cells, sometimes also including chemical analysis (Smaka-Kincl et al., 1996; Ohe et al., 2003; Žegura et al., 2009).

Utilizing plants instead of mammalian or microbial cells for testing methods has lots of benefits, like the similarity of plants and humans in the reaction to genotoxins, and the saving of expenses and time.

A test for the evaluation of genotoxicity makes use of *Allium cepa* root-tip cells, which are especially suitable therefore due to their large size and their small number of chromosomes. Recording morphological and cytogenetic factors allows drawing conclusions from the observed toxicity indicators, like the induction of micronuclei and chromosomal anomalies (Rank and Nielsen, 1998; Leme and Marin-Morales, 2009).

The *Allium cepa* test is suitable for the screening of the risks of coactions of various water contaminants like heavy metals, hydrophilic and lipophilic substances (Fiskesjö, 1985; Grover and Kaur, 1999; Rank et al., 2002; Caritá and Marin-Morales, 2008).

Performing the Allium test small bulbs (1.5 - 2.0 cm in diameter) of onion, *A. cepa*, (2n = 16) are used. First, the outer layers of the onion and the dry bottom plate of it have to be removed carefully. Six bulbs per water sample are immersed in distilled water for 48 hours.

The emerged root tips are removed from the bulb and checked for obvious morphological aberrations. For the test only the bulbs with a root length between 2 and 2.5 cm are taken. Three bulbs are immersed in each water sample. Tap water at a pH of 6.5 can serve as a

negative control (Fiskesjö, 1993, 1997), but has to be examined in its elements by atomic absorption spectrometry and its chloride ion concentration has to be evaluated by ion chromatography. Hydrogen peroxide can be utilized as a positive control.

After keeping the bulbs in the sample water for 24 hours, some root tips are cut from the bulbs, fixed in 3:1 ethanol:glacial acetic acid and stored overnight at 4°C. Afterwards, the root tips are rinsed with tap water and stained in aceto-carmine. Before amplifying the roots under the microscope they have to be mashed in 45 percent (v/v) acetic acid and each one placed on a microscope slide. Each bulb is observed under the bright-field microscope at a magnification of 1000-times.

For significant MI, about 6000 root cells, divided onto three microscope slides, have to be examined for each water sample. Results are obtained by reporting the observed chromosomal aberrations, including c-mitosis, laggards, chromosome breaks, anaphase bridges and stickiness, in 300 dividing cells.

To serve as an index of general toxicity the root lengths are measured after keeping the bulbs 72 hours in the sample water. The mitotic index and the root length index are obtained by calculating the percentage of the negative control.

Other possible markers that can be reported are modifications in root consistency or colour, swelling of the root due to c-tumours, and hooks or twists. (S. Radić et al., 2009)

8.2. DNA-adduct formation

The DNA-adduct formation observed in aquatic organisms is suitable parameter for the determination of genotoxic contaminant exposure. Therefore, the DNA has to be hydrolyzed enzymatically to 3`-monophosphates. After that, normal nucleotides are removed selectively to enrich the DNA adducts. Consequently, the adducts are labelled with [^{32}P] phosphate. The resulting ^{32}P-adducts are separated using TLC or HPLC. The radiolabeling makes it possible to use autoradiography and liquid scintillation counting, imaging analysis or a liquid scintillation analysis for the detection of adducts.

The ^{32}P-postlabeling technique is the most sensitive method for the detection of lots of different large hydrophobic compounds attached to DNA. The sensitivity of this technique enables to detect only one DNA-adduct that can be caused by polycyclic aromatic hydrocarbons in 10^9-10^{10} bases. (T. Ohe et al., 2004)

8.3. DNA strand breaks

DNA strand breaks often occur as pre-mutagenic lesions and therefore are sensitive indicators of genotoxic damage. By alkaline single cell gel electrophoresis (comet) assay (Mitchelmore et al., 1998) DNA strand breaks can be detected. This technique can be applied for any cell that has a nucleus. First, the nuclear DNA is unwound and a gel electrophoresis is performed at an alkaline pH larger than 13. This high pH reveals not only single-strand breaks but also other classes of DNA damage, like DNA protein cross-linking or alkali labile sites and incomplete DNA repair. As the DNA fragments move from the nucleus towards the anode the migration distance and the amount of DNA migration from a single nucleus indicates the extent of DNA damage. (T.Ohe et al, 2004)

9. Material and Methods

The Blue Rayon utilized for pre-concentration was purchased at,

FUNAKOSHI CO., LTD.
9-7 Hongo 2-Chome
Bunkyo-Ku, Tokyo 113
JAPAN

9.1 Preparation and analysis of samples from Danube and Mrhal

9.1.1. Preparation of standards

Benzo[h]quinoline, Phthalazine, and Quinoline were prepared as 200 μg mL^{-1}standard solutions. Fluorene, Phenanthrene, and Pyrene were prepared as 10 μg mL^{-1} standard solutions.

Therefore, the weighed amount was dissolved in a suitable solvent, water or acetonitrile, to prepare standard stock solutions of higher concentrations. Then the standard stock solutions were diluted to get 4 mL of each standard solution. An overview of the preparation of the standard solutions is given in tables 7.1-7.4.

Table 7.1: Preparation of standard stock solutions

standard stock solutions				
substance	**molar mass [g mol^{-1}]**	**weighed mass [g]**	**solvent**	**concentration of substance [μg/mL]**
Benzo[h]quinoline	179.22	0.001128	4 mL acetonitrile	282
Phthalazine	130.15	0.006398	3 mL water	2130
Quinoline	129.16	0.00094	3 mL water	313.3

Table 7.2: Dilution of standard stock solution to obtain 200 µg mL^{-1} standard solutions

standard 200 μg mL^{-1} (dilutions from standard stock solutions)				
substance	**molar mass [g mol^{-1}]**	**volume of standard stock solution [μL]**	**solvent**	**concentration of substance [μg/mL]**
Benzo[h]quinoline	179.22	2835	1165 μL acetonitrile	199.87
Phthalazine	130.15	375	3625 μL water	199.69
Quinoline	129.16	2550	1450 μL water	199.73

Table 7.3: Preparation of standard stock solutions

standard stock solutions				
substance	**molar mass [g mol^{-1}]**	**weighed mass [g]**	**solvent**	**concentration of substance [μg/mL]**
Fluorene	166.22	0.002054	10 mL acetonitrile	205.4
Phenanthrene	178.23	0.001240	10 mL acetonitrile	124.0
Pyrene	202.25	0.001354	10 mL acetonitrile	135.4

Table 7.4: Dilution of standard stock solution to obtain 10 µg mL^{-1} standard solutions

standard 10 μg mL^{-1} (dilutions from standard stock solutions)				
substance	**molar mass [g mol^{-1}]**	**volume of standard stock solution [μL]**	**solvent**	**concentration of substance [μg/mL]**
Fluorene	166.22	195	3805 μL acetonitrile	10.01
Phenanthrene	178.23	320	3680 μL acetonitrile	9.92
Pyrene	202.25	295	3705 μL acetonitile	9.99

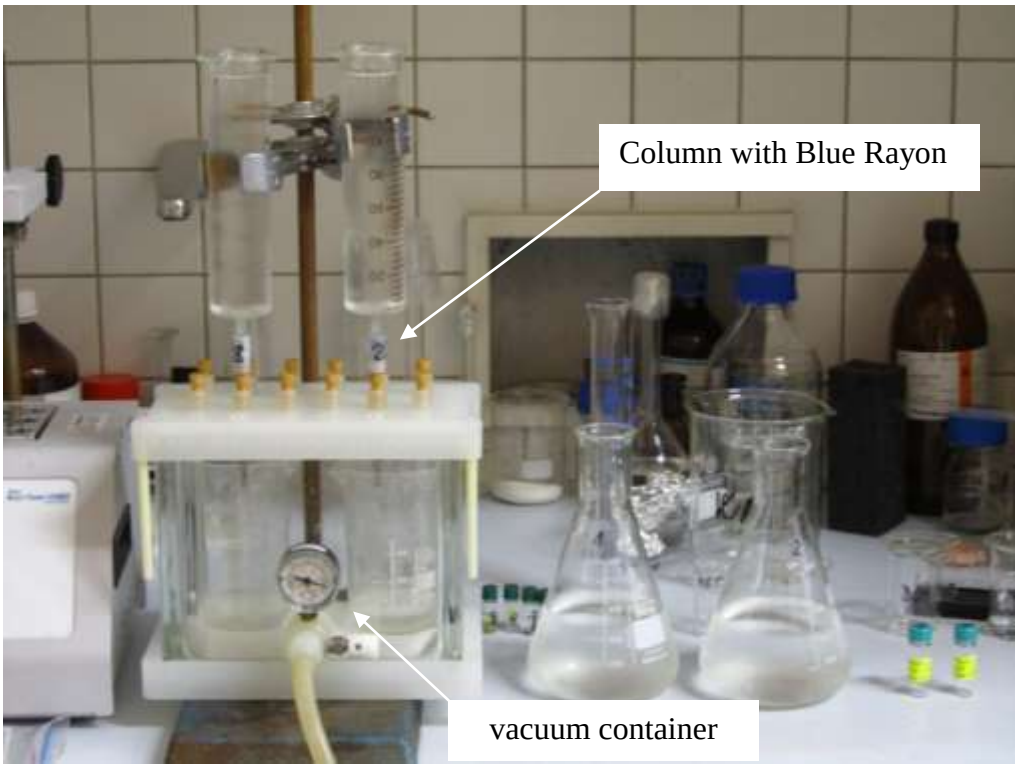
9.1.2. Preparation of elution mixture

For elution a mixture of ammonia:methanol (v:v = 1:50) was used.
Therefore, 8 mL of 25% ammonia were mixed with 92 mL of methanol.

9.1.3. Pre-concentration technique using Blue Rayon

For the use with Blue Rayon chromatographic columns were emptied and refilled with approximately 0.2 g Blue Rayon.
200 μL of each of the three standard solutions with a concentration of 10 μg mL^{-1} were put into 100 mL water.
To reach the same concentration of the other three standard solutions 10 μL of each of the standard solutions with a concentration of 200 μg mL^{-1} were put into another 100 mL water.
The pre-concentration set-up was based on applying a vacuum and using the gravitational force for moving the water sample through the column. The used apparatus is depicted in figure 19.

Figure 19: Assembly for extraction using Blue Rayon



100 mL of the standard solution containing three different contaminants were moved through a column containing the Blue Rayon. The procedure was repeated three times for each standard solution.

As a control 0.5 L tap water was moved through a column. This was repeated three times.

The water samples from Mrhal and Danube were examined in the same way as the tap water using 0.5 L sample water running through a column, repeating this three times.

After that the Blue Rayon was removed from the columns, immersed in the elution mixture and shaken thoroughly. Before the UHPLC-MS analysis the extracts were stored in the fridge.

9.2. Preparation and analysis of samples from Soběslav

The water samples from Soběslav were taken in the contaminated area close to the wood-preserving factory (Říha et al., 1993).

9.2.1. Pre-concentration technique using Blue Rayon

Approximately 0.3 g of Blue Rayon was stirred for four hours in 159 mL of water sample. Afterwards the Blue Rayon was removed from the water and dried carefully using some paper. The Blue Rayon was transferred to a 15 mL vial, which was filled with 10 mL of methanol:NH_4OH (v:v = 50:1) and shaken for 10 minutes. The extract solution was removed by a Pasteur pipette and collected in a 100 mL flask. The previous procedure was repeated.

The collected extract was evaporated by a rotation vacuum evaporator. Consequently, the extract was resuspended and unified in 3-times 1 mL of dichloromethane, which was again evaporated under a stream of nitrogen. For the analysis the concentrate was redissolved in 0.5 mL of toluene.

9.2.2. Analysis instrumentation

UHPLC UltiMate 3000 coupled with the HRMS LTQ Orbitrap XL (both Thermo Scientific, USA) has been used for the method development as well as for the sample analysis.

UHPLC UltiMate 3000

Injection volume: 5 μL
Column: Hypersil GOLD 2.1 × 150 mm, 3 μm (Thermo Scientific, USA)

Table 8: Gradient used for UHPLC instrumentation

Gradient: 30°C, flow: 0.3 mL min^{-1}	
Time [min]	**Mobile phase**
0	10% acetonitrile and 90% (0.1% acetic acid in water)
5	10% acetonitrile and 90% (0.1% acetic acid in water)
20	90% acetonitrile and 10% (0.1% acetic acid in water)
25	90% acetonitrile and 10% (0.1% acetic acid in water)
30	10% acetonitrile and 90% (0.1% acetic acid in water)

HRMS LTQ Orbitrap XL

Resolution: 60 000

Scan type: full scan

Scan ranges: 50-1000 m/z

Polarity: positive mode

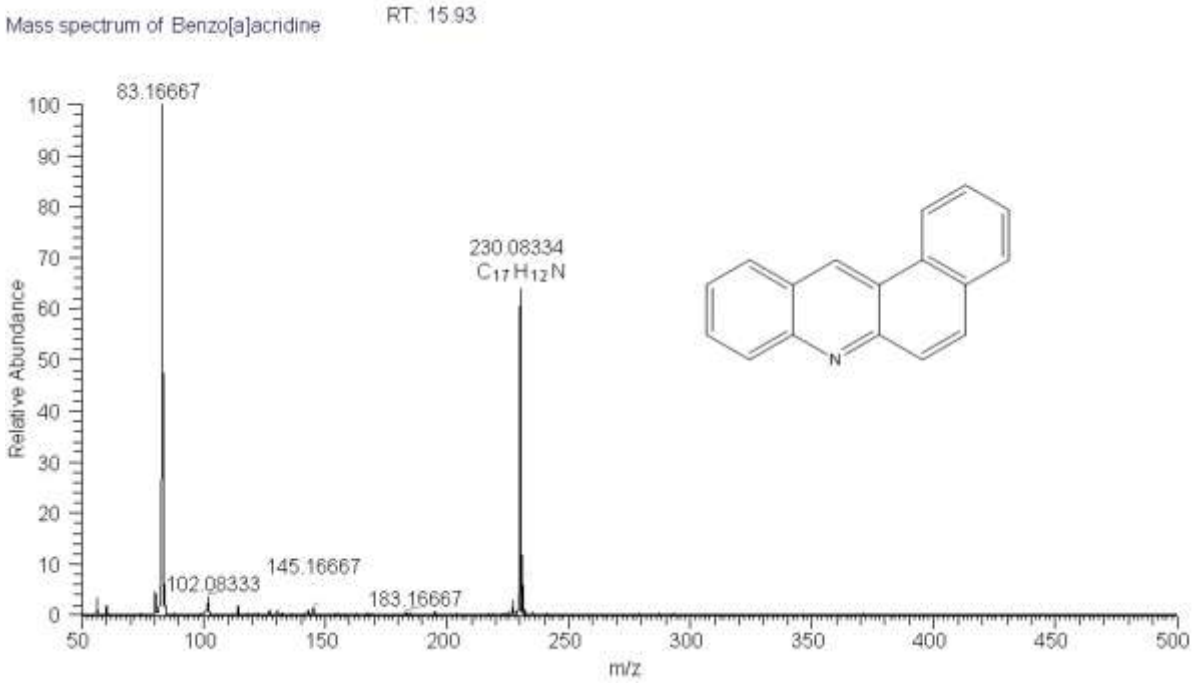
10. Results

The whole bachelor thesis is focused mainly on the literature search regarding occurrence and determination of organic compounds with mutagenic potential and assessment of water mutagenicity by different tests. It reveals from the literature survey that the most promising candidates for the extraction of organic pollutants from environmental waters are nitrogen containing polycyclic aromatic hydrocarbons with amino groups in the molecule with some exceptions (e.g. benzo[a]pyrene) (Hayatsu, 1992). A smaller part of the thesis, the laboratory experiment, was aimed to do a more detailed analysis of the described nitrogen containing polycyclic aromatic compounds and bare polycyclic aromatic hydrocarbons, which were expected to be found in the environmental waters, especially in the underground water of Soběslav. During the laboratory work the standard solutions of selected organic compounds with mutagenic potential were prepared. The pre-concentration of the chosen mutagens was done using Blue Rayon thereby also testing the sorption capacity and suitability of Blue

Rayon. Beside the water spiked with the pollutants, water from Danube river at three different locations in Austria, Mrhal pond and underground water samples from Soběslav were tested. Positive results were only found in the case of the water samples from Soběslav containing benzo[h]quinoline, benzo[a]acridine or benzo[b]acridine, the last two isomers could not be distinguished in the MS results.

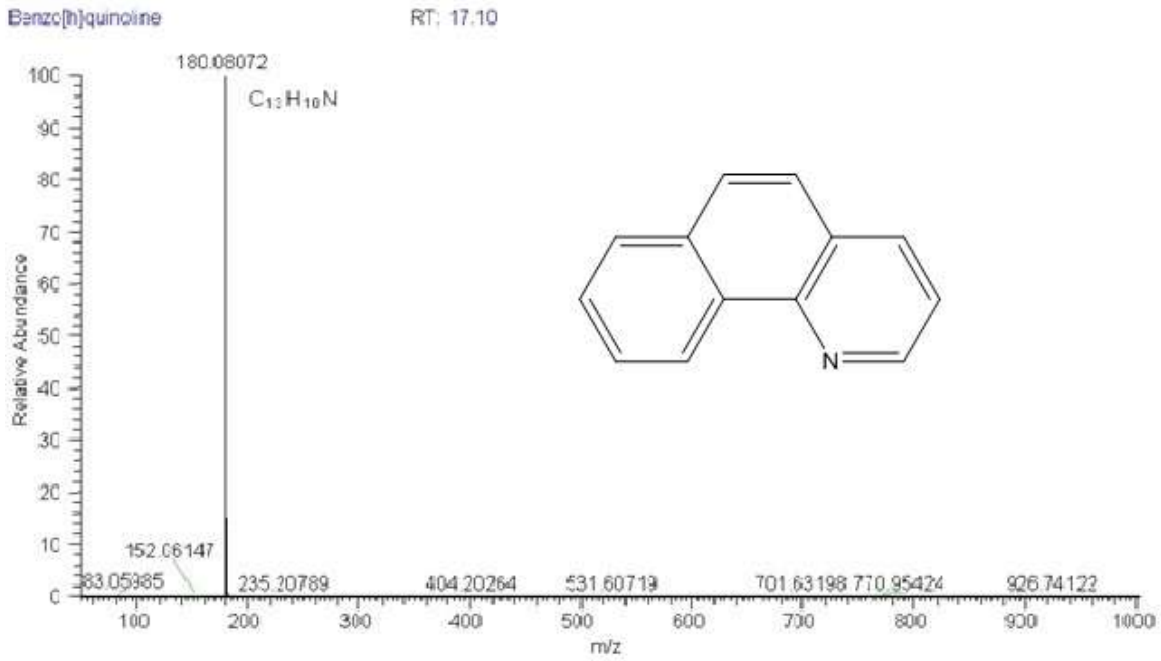
The mass spectrum shown in figure 20, resolves the molecular peak at m/z ratio of 230.08334, which corresponds to the singly positively charged protonated molecule of benzo[a]acridine.

Figure 20: Mass spectrum of benzo[a]acridine



The mass spectrum of benzo[h]quinoine, which is depicted in figure 21 shows the molecular peak at m/z ratio of 180.08072. The observed peak corresponds to the singly positively charged protonated molecule of benzo[h]quinoline.

Figure 21: Mass spectrum of benzo[h]quinoline



11. Conclusion

To summarize it up, an extensive literature research on the topic of chosen mutagenic polycyclic aromatic hydrocarbons and heterocyclic nitrogen containing aromatic compounds was conducted. The theoretical part of the thesis was focused on methods using Blue Cotton and Blue Rayon as a pre-adsorbents for the adsorption of diverse water pollutants. Another main part of theory contained the techniques for the categorization of the mutagenicity and carcinogenicity of polycyclic compounds. To assess the water mutagenicity Blue Rayon was utilized for pre-concentration later on analyzing the extracts by UHPLC-MS. The analysis of the water samples from the Danube and Mrhal showed to be free of the considered compounds. In the water samples from Soběslav mutagenic nitrogen containing heterocyclic compounds that are most probably caused by the wood processing factory in this area were detected.

12. References

Ames, B.N., et al., 1972. Carcinogens as Frameshift Mutagens: Metabolites and Derivatives of 2-Acetylaminofluorene and Other Aromatic Amine Carcinogens. *Proceedings of the National Academy of Science USA.*1972, 69, 11, pp. 3128-3132.

Ames, B.N., et al., 1973. Carcinogens are Mutagens: A Simple Test System Combining Liver Homogenates for Activation and Bacteria for Detection. *Proceedings of the National Academy of Science USA.*1973, 70, 8, pp. 2281-2285.

Aragão Umbuzeiro, G., et al., 2003. Investigating the sources of the mutagenic activity found in a river using the *Salmonella* assay and different water extraction prcedures. *Chemosphere.* 2004, 54, pp. 1589-1597.

Arimoto, S., et al., 1980 a. Inhibitory effect of hemin on the mutagenic activities of carcinogens. *Cancer Letters.*, 11, pp. 29-33.

Arimoto, S., et al., 1980 b. Inhibition of the mutagenicity of amino acid pyrolysis products by hemin and other biological pyrrole pigments. *Biochemical and Biophysical Research Communications.*, 92, pp. 662-668.

Bång, J., et al., 2002. Blue Chitin columns for the extraction of heterocyclic amines from cooked meat. *Journal of Chromatography A.* 2002, 977, pp. 97-105.

Britvić, S., et al., 2010. Detection of Mutagens and BaPMO Inducers in River Water Using the Blue Cotton adsorption Technique. *Environmental Toxicology.* 2012, 27, pp. 146-154.

Buhler, D.R., Wang-Buhler, J.-L., 1998. Rainbow trout cytochrome P450s: Purification, molecular aspects, metabolic activity, induction and role in environmental monitoring. *Comparative Biochemistry and Physiology.,* 121, pp. 107-137.

Busby, W.F., et al. 1989. Comparative lung tumorigenicity of parent and mononitropolynuclear aromatic hydrocarbons in the BLU:Ha newborn mouse assay. *Toxicology and Applied Pharmacology.,* 99, pp. 555-563.

Caritá, R., Marin-Morales, M.A., 2008. Induction of chromosome aberrations in the *Allium cepa* test system caused by the exposure of seeds to industrial effluents contaminated with azo dyes. *Chemosphere.* 2008, 72, pp. 722-725.

Churchley, J., 1994. Removal of dye waste colour from sewage effluent – the use of a full-scale ozone plant. *Water Science Technology.*, 30, pp. 275-284.

Claxton, L.D., et al., 1997. The development, validation, and analysis of *Salmonella* mutagenicity test methods for environmental situations. *Microscale Testing in Aquatic Toxicology.* CRC Lewis Publishers, Boca Raton, Florida, pp. 591-605.

Coelho, M., et al., 1992. Mutagenicity evaluation of industrial effluents by Ames assay. *Environmental and Molecular Mutagenesis.* 1992, 19, pp. 199-211.

Cruz, A., Buitron, G., 2001. Biodegradation of disperse blue 79 using sequenced anaerobic/aerobic biofilters. *Water Science & Technology.*, 44, pp. 159-166.

Devoret, R., 1981. Prophage lambda induction an inducible error-prone repair in *Escherichia coli*., in *W.E. Cohn (Ed.), Progress in Nucleic Acids Research and Molecular Biology, 46, Academic Press, New York,* pp. 251-263.

Ekici, P., et al., 2001. Degradability of selected azo dye metabolites in activated sludge systems. *Chemosphere.*, 44, pp. 721-728.

European Community, 1999. Proposal for a directive of the European parliament and of the Council amending for the nineteenth time Council Directive 76/769/eec relating to restrictions on the marketing and use of certain dangerous substances and preparations (azo colorants), com/99/0620 final- cod 99/0269. *Official Journal C 089 e, 28/03/2000*., pp. 67-69.

Fiskesjö, G., 1985. The Allium test as a standard in environmental monitoring. *Hereditas.* 1985, 102, pp. 99-112.

Fiskesjö, G., 1993. The *Allium cepa* in wastewater monitoring. *Environmental Toxicology and Water Quality.* 1993, 8, pp. 291-298.

Fiskesjö, G., 1997. Allium test for screening chemicals, evaluation of cytological parameters. In: Wang W., Gorsuch J.W., Hughes J.S., editors. *Plants for Environmental Studies.* 1997, New York, Lewis Publishers, pp. 308-333.

Freeman, H., et al., 1990. An approach to the design of non-mutagenic azo dyes: Analogs of the mutagen CI direct black 17. *Dyes Pigments.*, 13, pp. 5-77.

Gahr, F., et al., 1994. Ozonation - an important technique to comply with new German laws for textile wastewater treatment. *Water Science & Technology*, 30, pp. 255-263.

Garrison, A., Hill, D., 1972. Organic pollutants from mill persist in downstream waters. *Amer. Dysest. Rep.*, 61, pp. 21-25.

Graffi, A., et al., 1953. Studies on the tumour producing effects of different chemicals in combination with croton oil. *Arch Geschwulstforschung.*, 5, pp. 110-126 (in German).

Grover, I.S., Kaur, S., 1999. Genotoxicity of wastewater samples from sewage and industrial effluent detected by the Allium root anaphase aberration and micronucleus assays. *Mutation Research.* 1999, 426, pp. 183-188.

Hayatsu, H., et al., 1981. Inhibitory effect of the ether extract of human feces on activities of mutagens: Inhibition by oleic and linoleic acids. *Mutation Research.,* 81, pp. 287-293.

Hayatsu, H., et al., 1982. Adsorption of mutagens to cotton bearing covalently bound trisulfo-copper-phthalocyanine. *Mutation Research.* 1983, 119, pp. 233-238.

Hayatsu, H, 1992. Cellulose bearing covalently linked copper phthalocyanine trisulphonate as an adsorbent selective for polycyclic compounds and its use in studies of environmental mutagens and carcinogens. Journal of Chromatography. 1992, 597, pp. 37-56.

Houk, V.S., 1992. The genotoxicity of industrial wastes and effluents. *Mutation Research.* 1992, 277, pp. 91-138.

Huggins, C., Yang, N.C., 1962. Induction and extinction of mammary cancer. *Science.*, 137, pp. 257-262.

Huisman, O., D'Ari, R., 1981. An inducible DNA replication-cell division coupling mechanism in *E. coli*. *Nature (London).*, 290, pp. 797-799.

IARC, 1975. Some aromatic azo compounds. *IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans. International Agency for Research on Cancer, Lyon, France,* p. 357.

IARC, 1983. Polynuclear Aromatic Compounds, Part 1, Chemical, Environmental and Experimental Data. *IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans.*, 32, p. 431.

IARC, 1987. Overall Evaluations of Carcinogenicity: an Updating of IARC Monographs Volumes 1 to 42. *IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans.*, Supplement 7.

IARC, 1998. Certain Polycyclic Aromatic Hydrocarbons. *IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans.*, 3, p. 15.

IARC, 2005. Non-heterocyclic Polycyclic Aromatic Hydrocarbons and Some Related Exposures. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans.* 2010, 92.

Joachim, F., et al., 1985. Mutagenicity of azo dyes in the *Salmonella*/microsome assay using in vitro and in vivo activation. *Mutation Research.*, 156, pp. 131-138.

Kataoke, H., et al., 2000. Identification of mutagenic heterocyclic amines (IQ, Trp-P-1 and A alpha C) in the water of the Danube River. *Mutation Research.*, 466, pp. 27-35.

Kennaway, E.L., 1924a. On cancer-producing tars and tar-fractions. *Journal of Industrial Hygiene.*, 5, pp. 462-488.

Kennaway, E.L., 1924b. On cancer-producing factor in tar. *British Medical Journal*., i, pp. 564-567.

Kenyon, C.J., Walker, G.C., 1980. DNA-damaging agents stimulate gene expression at specific loci in *Escherichia coli*. *Proceedings of the National Academy of Science USA.,* 77, pp. 2819-2823.

Kira et al., 1995. A simple technique for monitoring mutagenicity and benzo(a)pyrene content in mussels and their ambient water. *Environmental Toxicology and Water Quality.*,10, pp. 167-172.

Kira, S., et al., 1996. Comparison of Techniques for Monitoring Water-Borne Polycyclic Mutagens: Efficiency of Blue Rayon, Sep-Pak C18, and a Biota, Corbicula, in Concentrating Benzo(a)pyrene in a Model Water system. *Bulletin of Environmental Contamination and Toxicology.* 1996, 57, pp. 278-283.

Kira, S., et al., 1997. Measurement of a Time-Weighted Average Concentration of Polycyclic Aromatic Hydrocarbons in Aquatic Environment Using Solid Phase Extraction Cartridges and a Portable Pump. *Bulletin of Environmental Contamination and Toxicology.* 1997, 58, pp. 879-884.

Kira, S., et al., 1998a. Measurement of a Time-Weighted Average Concentration of Polycyclic Aromatic Hydrocarbons in Seawater: An Improved Procedure of Blue Rayon Hanging Technique for Monitoring Benzo(a)pyrene. *Marine Environmental Research.* 1998, 46, 1-5, pp. 267-271.

Kira, S., et al., 1998b. Detection of Benzo(a)pyrene and Mutagenicity in Water of Lake Baikal (Russia) and Rivers in Okayama (Japan) Using the Blue Rayon Method: A Simplified Handling and Transportation of Samples from Remote Sites. *Environmental Toxicology.*, 14, pp. 279-284.

Komatsu, T., Kawai, H., 1992. Measurement of Time-Averaged Intensity of water Motion with Plaster Balls. *Journal of Oceanography.*1992, 48, pp. 353-365.

Kummrow, F., et al., 2003. Comparison of the mutagenic activity of XAD4 and blue rayon extracts of surface water and related drinking water samples. *Mutation Research.* 2003, 541, pp. 103-113.

La Voie, E.J., et al., 1981. Mutagenicity, tumor-initiating activity, and metabolism of methylphenanthrenes. *Cancer Research.*, 41, pp. 3441-3447.

Leme, D.M., Marin-Morales, M.A., 2009. *Allium cepa* test in environmental monitoring: a review on ist application. *Mutation Research.* 2009, 682, pp. 71-81.

Little, J.W., Mount, D.W., 1982. The SOS regulatory system of *Escherichia coli*. *Cell.*, 29, pp. 11-22.

Maguire, J.R., Tkacz, R.J., 1991. Occurrence of dyes in the Yamaska river, Quebec. *Water Pollut. Res. J. Can.*, 26, pp. 145-161.

McCann, J., et al., 1975. Detection of carcinogens as mutagens in the *Salmonella*/microsome test: Assay of 300 chemicals. *Proceedings of the National Academy of Science USA.*1975, 72, 12, pp. 5135-5139.

Mitchelmore, C.L., Chipman, J.K., 1998. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. *Mutation Research.*, 399, pp. 135-147.

Mortelmans, K., Zeiger, E., 2000. The Ames *Salmonella*/microsome mutagenicity assay. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis.* 2000, 455, 1-2, pp. 29-60.

Nebert, D.W., Gelboin, H.V., 1968. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. *Journal of Biological Chemistry.*1968, 243, pp. 6242-6249.

Nukaya, H., et al., 2001. Identification of 2-[2-(acetylamino)-4-amino-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2h-benzotriazole (PBTA-4) as a potent mutagen in river water in Kyoto and Aichi Prefectures, Japan. *Mutation Research.*, 492, pp.73-80.

Oda, Y., et al., 1985. Evaluation of the new system (umu-test) for the detection of environmental mutagens and carcinogens. *Mutation Research/Environmental Mutagenesis and Related Subjects.* 1985, 147, 5, pp. 219-229.

Ohe, T., et al., 2003. Mutagenic characteristics of river waters flowing through large metropolitan areas in North America. *Mutation Research Genetic Toxicology and Environmental Mutagenesis.* 2003, 534, pp. 101-112.

Ohe, T., et al., 2004. Mutagens in surface waters: a review. *Mutation Research.*2004, 567, pp. 109-149.

Ono, Y., et al., 2000. Identification of a carcinogenic heterocyclic amine in river water. *Water Research.*, 34, pp. 890-894.

Osemwengie, L.I., Steinberg, S., 2001. On-site solid-phase extraction and laboratory analysis of ultra-trace synthetic musks in municipal sewage effluent using gas chromatography - mass spectrometry in the full scan mode. *Journal of Chromatography A.*, 932, 1-2, pp. 107-118.

Pagga, U., Brown, D., 1986. The degradation of dyestuffs, part II. Behaviour of dyestuffs in aerobic biodegradation tests. *Chemosphere*, 15, pp. 479-491.

Parrott, J.L., et al., 2000. Isolation of MFO inducers from tissues of white suckers caged in bleached kraft mill effluent. *Chemosphere.*, 41, pp. 1083-1089.

Quillardet, P., et al., 1982. SOS Chromotest, a direct assay of induction of an SOS function in *Escherichia coli* K12 to measure genotoxicity. *Proceedings of the National Academy of Science USA.*, 79, pp. 5971-5975.

Quillardet, P., Hofnung, M., 1984. The SOS Chromotest, a colorimetric bacterial assay for genotoxins: procedures. *Mutation Research.* 1985, 147, pp. 65-78.

Radić, S., et al., 2009. The evaluation of surface and wastewater genotoxicity using the *Allium cepa* test. *Science of the Total Environment.* 2010, 408, pp. 1228-1233.

Radman, M., 1975. Phenomenology of an inducible mutagenic DNA repair pathway in *Escherichia coli*: SOS repair hypothesis, in: L. Prakash, F. Sherman, M. Miller**, C.** Laurence and H.W. Taylor (Eds.). *Molecular and Environmental Aspects of Mutagenesis, Thomas, Springfield, IL*, pp. 128-142.

Rank, J., Nielsen, M.H., 1998. Genotoxicity testing of wastewater using the *Allium cepa* anaphase-telophase chromosome aberration assay. *Mutation Research.*1998, 418, pp. 113-119.

Rank, J., et al., 2002. Genotoxicity of maleic hydrazide , acridine and DEHP in *Allium cepa* root cells performed by two different laboratories. *Hereditas.* 2002, 136, pp. 13-18.

Rehana, Z., et al., 1995. Mutagenic activity of the ganges water with special reference to the pesticide pollution in the river between Kachla and Kannauj (U.P.), India. *Mutation Research.*, 343, pp. 137-144.

Rehana, Z., et al., 1996. Genotoxicity of the Ganges water at Narora (U.P.), India. *Mutation Research.*, 367, pp. 187-193.

Reid, T.M., et al., 1984. Mutagenicity of azo dyes following metabolism by different reductive/oxidative systems. *Environmental Mutagenesis.*, 6, pp. 705-717.

Říha, V., et al., 1993. Microbiological, chemical and toxicological characterization of contaminated sites in Czechoslovakia. *The Science of the Total Environment*.pp. 185-193.

Roe, F.J.C., 1962. Effect of phenanthrene on tumour-initiation by 3,4-benzopyrene. *British Journal of Cancer.*, 16, pp. 503-506.

Ross, J.A., et al., 1995. Adenomas induced by polycyclic aromatic hydrocarbons in strain A/J mouse lung correlate with time-integrated DNA adduct levels. *Cancer Research.*, 55, pp. 1039-1044.

Sakamoto, H., et al., 1996. Evaluation of blue-chitin column, blue rayon hanging, and XAD-resin column Techniques for concentrating mutagens from two Japanese rivers. *Mutation Research.* 1996, 371, pp. 79-85.

Salaman, M.H., Roe, F.J.C., 1956. Further tests for tumour-initiating activity: N,N-Di-(2-chloroethyl)-p-aminophenyl-butyric acid (CB1348) as an initiator of skin tumour formation in the mouse. *British Journal of Cancer.*, 10, pp. 363-378.

Sanchez, P., et al., 1988. Toxicity assessment of industrial effluents from S. Paulo state using short-term microbial assays. *Toxic Assessment.* 1988, 3, pp. 55-80.

Scribner, J.D., 1973. Tumor initiation by apparently noncarcinogenic polycyclic aromatic hydrocarbons., *Journal of the National Cancer Institute.*, 50, pp. 1717-1719.

Shiozawa, T., et al., 1999. Mutagenic activity of 2-phenylbenzotriazole derivatives related to a mutagen, PBTA-1, in river water. *Mutation Research.*, 442, pp. 105-111.

Smaka-Kincl, V., et al., 1996. The evaluation of waste, surface and ground water quality using the *Allium cepa* test procedure. *Mutation Research.* 1996, 368, pp. 171-179.

Steiner, P.E., 1955. Carcinogenicity of multiple chemicals simultaneously administered. *Cancer Research.*, 12, pp. 632-635.

Takamura-Enya, T., et al., 2002. Identification of a new mutagenic polychlorinated biphenyl derivative in the Waka River, Wakayama, Japan, showing activation of an aryl hydrocarbon receptor-dependent transcription. *Chemical Research in Toxicology.*, 15, pp. 419-425.

United Nations University, 2003.
www.inweh.unu.edu/biology447/modules/module1/1g_t3.htm accessed on 01/04/2003, United Nations

Venturini, S., Tamaro, M., 1979. Mutagenicity of anthraquinone and azo dyes in Ames' *Salmonella* typhimurium test. *Mutation Research.*, 68, pp. 307-312.

Walker, G.C., 1984. Mutagenesis and inducible responses to DNA damage in *Escherichia coli*. *Microbiology Review.*, 48, pp. 60-93.

Warshawsky, D., et al., 1993. Factors affecting carcinogenic potential of mixtures. *Fundamental applied Toxicology*, 20, pp. 376-382.

Weber, E.J., Adams, R.L., 1995. Chemical and sediment-mediated reduction of the azo dye disperse blue 79. *Environmental Science & Technology.*, 29, pp. 1163-1170.

Wenzel-Hartung, R., et al., 1990. Evaluation oft he carcinogenic potency of four environmental polycyclic aromatic compounds following intrapulmonary application in rats. *Experimental Pathology.*, 40, pp. 221-227.

White, P.A., and Rasmussen, J.B., 1998. The genotoxic hazards of domestic waters in surface waters. *Mutation Research.*, 410, pp. 223-236.

White, P.A., et al., 1999. A mass balance of surface water genotoxicity in the Providence River (Rhode Island, USA). *Environmental and Molecular Mutagenesis.*, 33, Supplement 33, p. 68.

Wislocki, P.G., et al., 1986. Tumorigenicity of nitrated derivatives of pyrene, benz[a]anthracene, chrysene and benzo[a]pyrene in newborn mouse assay. *Carcinogenesis.*, 7, pp. 1317-1322.

Witkin, E.M., 1976. Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*. *Bacteriological Review.*, 40, pp. 869-907.

Wood, A.W., et al., 1979. Mutagenicity and tumorigenicity of phenanthrene and chrysene epoxides and diol epoxides. *Cancer Research.*, 39, pp. 4069-4077.

Žegura, B., et al., 2009. Combination of in vitro bioassays for the determination of cytotoxic and genotoxic potential of wastewater, surface water and drinking water samples. *Chemosphere.* 2009, 75, pp. 1453-1460.