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Catecholamine Homeostasis in *Tetrahymena* species and High Throughput Toxicity Testing of Selected Chemicals and Ultrafine Particles

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This thesis is dedicated
to
my beloved parents

Brief Contents

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Summary

The purpose of this investigation is to determine the toxic effect of catecholamines, 5-fluorouracil, perfluorinated chemicals and nanoparticles in unicellular ciliates protozoan, *Tetrahymena* species based on 96 well plate formats. The test substances are added in various concentrations into exponentially growing cultures. The reduction of cell growth with relation to an untreated control is regarded as a measure of the toxicity of those compounds. Catecholamine degradation and metabolism are determined employing HPLC. In addition fluorescent microscopy was used for the identification of dopamine receptors in *Tetrahymena thermophila*.

Catecholamines exerted moderate acute toxicity to *Tetrahymena* cells. L-DOPA, DOPAC and noradrenaline were demonstrated the EC_{10} of 0.61, 5.95 and 121 µmol after 24 h exposure. In addition these compounds showed EC_{20} of 6.39, 19.7 and 246 µmol at the same exposure time. Unfortunately, effective concentrations of dopamine and adrenaline were not claimed due to the insignificant cell growth inhibition. On the other hand, all the test compounds presented effective concentrations after 48 h exposure. Wherein, dopamine and L-DOPA showed higher toxic potential due to the demonstration of lower effective concentrations. EC_{10} of dopamine and L-DOPA were found to be 2.55 and 3.19 µmol respectively and EC_{20} to be 7.18 and 5.07 µmol respectively after 48 h exposure. Catecholamines are highly degradable in the PPY-medium due to the oxidizing environment during the period of incubation. The degradation rates of dopamine, adrenaline and noradrenaline were found to be 97.6%, 88% and 85% respectively. However, the degradation rate could be replenished at a small scale by the addition of *Tetrahymena pyriformis* cell due to the natural synthesis of catecholamines. *Tetrahymena* produce catecholamines and liberate them into the culture medium which accumulated over time. Noradrenaline showed the highest degree of accumulation of 100 nmol after 24 h. However, dopamine and adrenaline elicited higher concentrations of 21.7 and 7.03 nmol respectively after 32 h exposure. Interestingly, the exogenous exposure of catecholamines to the cells caused the depletion of natural noradrenaline synthesis even at the use of very low physiological concentration (0.12 ppm). Dopamine exerted the higher effect on depleting noradrenaline synthesis. Consequently, the treatment of dopamine and L-DOPA at higher concentration (8.0 ppm) in 96-well plates poses strong excitation in the cells and liberated a new metabolite *in vivo* while adrenaline, noradrenaline, DOPAC did not provoke the production of the same metabolite. This

metabolite is relatively non-polar as compared to noradrenaline, adrenaline and dopamine and eluted later through the reverse phase C-18 column.

A UPLC-MS method has been developed for the separation and detection of noradrenaline, adrenaline, dopamine and new metabolite in similar order of retention time as for HPLC-ED. Reverse phase C-18 column was used for both tools. The metabolite demonstrated the molecular weight of $[M+H]^+$, 151 m/z using positive electrospray ionization. As a consequence the metabolite was also analyzed employing FTICR-MS for determination of molecular formula. FT-MS is specially characterized by its ultrahigh resolution in broadband measurements and high mass accuracy. This tool could also identify the same metabolite in identical mass of UPLC-MS. Therefore, the assignment of metabolite peak at $[M+H]^+$, 151.08632 m/z explored the molecular formula to X. However, due to the small time frame this study could not elucidated the structure of new metabolite. Nevertheless, it proposed a possible name of metabolite depending on literature survey.

This study also revealed the dopamine receptor in *Tetrahymena thermophila*. Two types of dopamine receptor exist in the cell like D1 type and D2 type. Here, only cellular localization of D1 type dopamine receptor was explored in *Tetrahymena* by the use of a fluorescent dopaminergic specific agonist, SKF-38393. This agonist exerted saturation binding after 3 h. The specific binding of SKF-38393 to D1 receptor was confirmed by Dl-specific antagonist, SCH 23390 which prevents the binding of SKF-38393. On the other hand, D2 selective antagonist, spiperone could not inhibit the binding of the SKF-38393. Thus, clearly claimed the specific binding of SKF-38393 to D1 type receptor, and consequently focuses that SKF-38393 did not play cross reaction with D2 type receptor. However, due to the exposure of agonist in the *Tetrahymena* cell causes the receptor internalization from plasma membrane into endosome. The receptor internalization was confirmed with the photography of laser scanning microscope. D1 receptor protein is abundant in endosome and also poorly distributed in the transport vacuoles which originated from rough ER.

5-fluorouracil inhibits cell growth and exhibits acute toxicity to fresh water green microalgae, *Scenedesmus vacuolatus* and the ciliated protozoan, *Tetrahymena pyriformis*. 5-fluorouracil magnify in the algal cell which reveals bioaccumulation factor of 18.4×10^3. This bioaccumulation of 5-FU was ascertained by the application of radio-labelled [2-^{14}C] 5-FU compound at laboratory scale. That compound not only accumulates in the algae cell but it is

also integrated within its genome. Radio-labelled 5-fluorouracil was detected in both DNA and RNA of algae with percentages of 0.41 and 0.59 respectively of the initially applied radioactivity. These labelled algae genome obtained from labelled 5-FU and transferred into another organism when introduced into the *Tetrahymena* culture. The second organism fed these labelled genome and showed radioactivity in the whole cell. The presence of radioactive 5-FU in the second organism claimed an emerging question whether the 5-FU reintegrates again into the genome of *Tetrahymena* or not. But radioactivity in the genome of predator organism was not identified. This study could be regarded as a source of introduction of this toxic substance to the natural environment and its risk of harm to the aquatic microbial ecology.

This study also demonstrated the growth inhibition and lactate dehydrogenase (LDH) leakage of ciliates protozoan, *Tetrahymena pyriformis* to PFOA and PFOS. These compounds affected the growth of *Tetrahymena* at very low concentrations, but significant growth inhibition was observed only in samples exposed to concentrations higher than 25 mg/L. PFOS was more significant as compared to PFOA. The EC_{50} value of PFOS was established to 93 and 72 ppm after 24 and 48 hour incubation respectively. However, PFOA exerted the EC_{20} to 77.6 and 64.5 ppm after 24 and 48 hour incubation respectively at same concentration of PFOS. Furthermore, these compounds induce cell membrane damage by partitioning in the lipid bilayer and consequently cause the release of LDH from cytoplasm into the culture medium. PFOA produce elevated LDH leakage than the PFOS which is counterpart of growth inhibition in *Tetrahymena.* On the other hand, the perfluorinated telomere alcohols (4:2 FTOH, 6:2 FTOH, 8:2 FTOH and 10:2 FTOH) neither demonstrated the cell growth inhibition nor membrane damage. Surprisingly, perfluorinated alcohols induce damage of macronucleus of *Tetrahymena* cell. Macronucleus damage was visualized by the use of acridine orange dye. This dye binds with DNA and subsequently emits fluorescents light at 488 nm which was observed with fluorescent microscopy.

In this research TiO_2 nanoparticles ($\leq$ 10 nm) were also studied with *Tetrahymena* population by means of counting their number by a Neubauer counting chamber after 0, 5, 24, 29 and 28 hours. TiO_2 did not clearly exhibit toxicity even in very high concentrations of nanoparticles applied in the cultivation medium (up to 400 mg/L). Microscopic studies of TiO_2 revealed that *Tetrahymena* cells ingest TiO_2 particles and form food vacuoles wherein they store until exocytose into the medium. Interestingly the TiO_2 undergoes agglumerization and are later

released as bigger aggregates. However, the aggregation of particles occurs randomly with *Tetrahymena* growth over time. Therefore, *Tetrahymena* cell can act as a factor for the aggregation of nanoparticles in the aquatic environment.

List of publications

List of research publications

1. **Ud-Daula A.**, Pfister G., Schramm K-W., **2009**. 5-Fluorouracil accumulation in green microalgae and its biogenetic transfer into ciliate protozoan. *CEMEPE*, 1, 239-243 (accepted).
2. **Ud-Daula A.**, Pfister G., Schramm K-W., **2008**. ISTA13-Catecholamine toxicity and metabolism in the ciliated protozoan, *Tetrahymena pyriformis*. *Journal of Environmental Toxicology,* PMID: 19051280 (In press).
3. **Ud-Daula A.**, Pfister G, Schramm K-W, **2008.** Growth inhibition and biodegradation of catecholamines in the ciliated protozoan *Tetrahymena pyriformis*. *Journal* Environronmental Science and Health A Tox Hazard Subst Environ Eng., 8 (14), 1610-7.
4. Zezulka Š., **Ud-Daula A.,** Fiedler S., Schramm K.-W., **2007**. The effect of perfluorinated surfactants on the growth of ciliate protozoan *Tetrahymena pyriformis.*.

List of contribution in international scientific conference and symposium

1. Baykal S.B., **Ud-Daula A**., Schramm K-W., **2008.** Fate and effect of TiO_2 and Fe_3O_4 nanoparticles in the aquatic environments. *3rd International Conference on the Environmental Effects of Nanoparticles and Nanomaterials.* ID. 30, September 15-16, University of Birmingham, **UK.**
2. **Ud-Daula A.**, Pfister G., Schramm K-W., **2007**. Genetic biotransfer of 5-fluorouracil and its toxicity risk in the aquatic environments. *Mediterranen Scientific Association of Environmental Protection (MESAEP)* 14th International Symposium. p 437, October 10-14, Sevilla, **Spain.**
3. **Ud-Daula A.,** Pfister G., Schramm K-W., **2007**. Catecholamine toxicity and metabolism in the ciliated protozoan, *Tetrahymena pyriformis.* 13th International Symposium on Toxicity Assessment (ISTA).p 103, August 19-24, Toyama, **Japan**.
4. **Ud-Daula A.**, Stocker A., Pfister G., Schramm K-W., **2007**. High throughput method of catecholamine metabolism in the ciliated protozoan, *Tetrahymena pyriformis*. *Society for Environmental Toxicology and Chemistry (SETAC)* Europe 17th Annual Meeting. ID. 2105, May 20-24, Porto, **Portugal**.
5. **Ud-Daula A.**, Stocker A., Pfister G., Schramm K-W., **2005**. Toxicity Study of catecholamines against *Tetrahymena*. *Munich Environmental Microbiology (MEM_05)* Meeting. p 33, November 29, Munich, **Germany.**

Abbreviation and acronyms

A	:	Adrenaline
AAAD	:	L-aromatic amino acid decarboxylase
AO	:	Acridine orange
AS	:	Analytical solution
AS50	:	Auto sampler 50
BH4	:	L-erythro-tetrahydrobiopterin
BP	:	Base pair
cAMP	:	Cyclic adenosine mono-phosphate
CAs	:	Catecholamines
CMC	:	Critic micelle concentration
CMR	:	Carcinogenic, mutagenic and reprotoxic
COMT	:	Catechol-o-methyltransferase
DA	:	Dopamine
DHBA	:	3,4-dihydroxybenzylamine
DβH	:	Dopamine beta hydroxylase
DHFU	:	5,6-dihydrofluorouracil
DNA	:	2-Deoxyribonucleic acid
DOPA	:	3,4-dihydroxyphenyl alanine
DOPAC	:	3,4-dihydroxyphenyl acetic acid
DPD	:	Dihydropyrimidine dehydrogenase
DPM	:	Disintegration per minute
dTMP	:	Deoxythymidine monophosphate
dTMP	:	Deoxythymidine monophosphate
dUMP	:	Deoxyuridine monophosphate
EC	:	Effective concentration
ED	:	Electrochemical detector
ER	:	Endoplasmic reticulum
FdUMP	:	Fluorodeoxyuridine monophosphate
FdUTP	:	Fluorodeoxyuridine monophosphate
FTICR-MS	:	Fourier Transform Ion Cyclotron Resonance-Mass Spectroscopy
FTOH	:	Perfluorotelomer alcohol
FUMP	:	Deoxyuridine monophosphate
FUR	:	5-Fluorouridine
5-FU	:	5-Fluorouracil
FUMP	:	5-fluorouridine-5´-monophosphate
FUTP	:	Deoxythymidine monophosphate
FW	:	Fresh weight
GP40	:	Gradient pump 40
GPCRs	:	G-protein coupled receptors
HPLC	:	High performance liquid chromatography
IS	:	Internal standard
LC	:	Liquid chromatography
LDH	:	Lactate dehydrogenase
LPO	:	Lipid per-oxidation
LSM	:	Laser scanning microscope
MAO	:	Monoamine oxidase
MRM	:	Multiple reactions monitoring
MS	:	Mass spectrometry
NA	:	Noradrenaline

NMs	:	Nanomaterials
NPs	:	Nanoparticles
OD	:	Optical density
OECD	:	Organization for economic co-operation and development
OPRT	:	Orotate phosphoribosyltransferase
PBS	:	Phosphate buffer saline
PFCs	:	Perfluorinated compounds
PFOA	:	Perfluorooctanoic acid
PFOS	:	Perfluorooctane sulfonate
PGG2	:	Prostaglandin G2
PGH2	:	Prostaglandin H2
PM	:	Particulate matter
PNMT	:	Phenylethanolamine-N-methyl-transferase
PPM	:	Pers per million
PPY	:	Proteose peptone yeast extract
PRPP	:	5-Phosphoribosyl-1-Pyrophosphate
PTFE	:	Polytetrafluoroethylene
REC	:	Recovery
ROS	:	Reactive oxygen species
RPM	:	Rotation per minute
RR	:	Ribonucleotide reductase
RT	:	Retention time
SCH-23390	:	7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine
SD	:	Standard deviation
SI	:	Substrate inhibition
SKF-38393	:	R(+)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol
SML	:	Surface monolayer
SPE	:	Solid phase extraction
SSA	:	Specific surface area
TEA^-	:	Tetraethyl ammonium
TIC	:	Total ion current
TK	:	Thymidine kinase
TMO	:	Tyrosine-3- monooxygenase
TOF	:	Time of flight
TP	:	Thymidine phosphorylase
TS	:	Thymidine synthase
UK	:	Uridine kinase
UPLC	:	Ultra Performance liquid chromatography
WWTP	:	Waste water treatment plant

1. Introduction

In the present thesis, a high throughput testing system was developed for catecholamines, 5-flurouracil, perfluorinated compounds and nanoparticles against single cell ciliate protozoa, *Tetrahymena* species. There are many compounds studied in the newly developed high throughput system. However, it is the first time reporting about catecholamine and other compounds which have been studied in 96-well plates in *Tetrahymena pyriformis.* Catecholamines are a class of biogenic monoamines which act both as neurotransmitter and neurohormone. The main representative members of catecholamines are dopamine, noradrenaline and adrenaline. Dopamine is the key member which is used for Parkinson disease as therapy.

On the other hand, 5-fluorouracil (5-FU) is used as anticancer drug and enters into the aquatic environments through the sewage network. Perfluorinated compounds (PFCs) are persistent in the environment and have emerged as a new class of global environmental pollutants. They originate from different surfactants, refrigerants, polymers and pharmaceuticals and finally end up into the ponds; rivers; ocean etc. Nanoparticles (NPs) are increasingly used in industrial and household applications and very likely lead to the release of such compounds in the environments. Therefore, the study of these compounds and their effect on aquatic microorganisms is required in prior basis. However, the adverse effect of these kind of compounds especially catecholamines and 5-fluorouracil are broadly examined in animal cell lines. Here, these compounds are tested and described on their toxicity using 'animal free cell' in 96 well plates.

Catecholamines are known to cause toxicity to neuroblastoma cells. The exogenous exposures of catecholamine at low concentration produce cell injury and deplete the number of cells through the oxidation of its catechol moiety (Rosenberg, 1988). In previous studies it was reported that dopamine oxidation readily produce reactive oxygen species (ROS) and reactive quinones via spontaneous, enzyme-catalyzed or metal enhanced reactions (Stokes *et al*., 1999; Cyr *et al.*, 2003; Stokes *et al*., 2000). However, DA-quinone itself is directly cytotoxic and genotoxic in addition to its significant role in generating ROS (Stokes *et al.,* 1999). Indeed, dopamine oxidation induces programmed cell death or apoptosis (Walkinshaw and Waters, 1995; Melamed *et al*., 1994). It is documented that generation of ROS during dopamine oxidation may be one of the causes of the death of dopaminergic neurons in Parkinson diseases (Spina and Cohen, 1989). However, there is no enzymatic system yet known for the

generation of either noradrenaline or adrenaline semiquinone. Nevertheless, noradrenaline and adrenaline perform oxidation with the formation of toxic quinone by the reaction with non-physiological one-electron acceptor (Ole *et al*., 2006). Thus, not only dopamine but also noradrenaline and adrenaline can promote the degeneration of noradrenergic neurons. Vertebrate cell line is the only system used for catecholamine analysis. This study first time investigated and explored the effect and homeostasis of catecholamines to *Tetrahymena* in 96 well plates. Many studies claimed that the *Tetrahymena pyriformis* capable of the synthesising of biogenic monoamines (Le Roith and Roth, 1985; Brizzi and Blum 1970; Goldman *et al*., 1981; Gundersen and Thompson, 1983; Naokuni and Kanji, 1993). Consequently these studies imposed only the quantification and metabolism of biogenic monoamines in *Tetrahymena.* The study on toxicity of catecholamine on that cell is also of important ubiquitous concern in the biological kingdom. Literature reported that catecholamines can produce toxicity to the same cell from where they synthesized e.g. neurone cell (Cyr *et al*., 2003). Therefore, on this respect *Tetrahymena* cell was considered for describing adverse effect of catecholamines. However, the external use of low concentration of catecholamines can substantially affect its metabolism and liberates an unknown metabolite.

In particular, the external use of catecholamine can excite the cell and induce it to produce further chemical signalling. This chemical signal may have the ability to generate new metabolites in the cell. However, metabolites are the most sensitive diagnostic indicators or biomarkers which are increasingly used in environmental and medical diagnosis. Only a very small fraction of the existing low molecular substances can be roughly quantified. In this research a MS technique was established as a powerful research tool in clinical as well as model organisms useful for in vitro studies of metabolites. The formation of new compounds is exemplified using selected protozoan species, e.g. *Tetrahymena* in presence and absence of catecholamines. These compounds are analyzed using High Performance Liquid chromatography (HPLC) connected with electrochemical detection. It is considered as a convenient tool for this analysis. Liquid chromatography connected with electrochemical detection (LC-ED) is the most widely used technique for catecholamine analysis (Hay and Mormède, 1997; Volin, 1994) and the identification of new metabolites. Electrochemical detection is much more sensitive as compared to other detectors which perform trace analysis of catecholamine detection present in the cell extract matrix.

In this research work, the mass spectrometry (MS) was used to characterize metabolites. MS acts as highly specific detector which allows peak identification even in complex sample matrices. It also gives structural information which describes each analyte. This tool increases the reliability of the identification of analytes. It also provides a highly selective, sensitive, and robust technique for detection and quantization of a wide variety of compounds after an appropriate analytical separation (Wu and Furlanut, 1997; Niessen, 1999). The most relevant aspect of this approach is the drastic reduction in method-development time for readily ionizable analytes. This can be possible due to the dramatic improvement in selectivity offered by tandem mass spectrometry (Lee and Kerns, 1999). It is now possible to develop and apply a bio-analytical method after a development time of only a few days. The use of electrospray ionization has been discussed intensively in the analysis and identification of catecholamines (Hirabayashi *et al*., 1995; Rudewicz and Straub, 1986; Kerwin, 1996). Liquid chromatography coupled to mass spectrometry (LC/MS) could be considered as a well-accepted technique which identifies unknown metabolites in environmental samples (Hunter *et al*., 2004; Petrovic and Barcelo, 2006; Uebori and Imamura, 2004). Wenlin *et al*., (2000) identified catecholamines and their metabolites in rat plasma by a compatible ESI-LC-MS-MS with positive ionization. A sensitive LC–MS–MS analytical technique has been developed for urinary catecholamines after a specific sample purification step (Kushnir, 2002). In this study, UPLC-MS (Q-Tof 2 MS) with electrospray ionization was considered as complementary approach for the determination of the molecular weight of unknown dopamine metabolites. This method could provide an analytical advantage, including excellent selectivity of metabolite detection with respect to the same pattern of retention time of HPLC-ED. The choice of extraction conditions is redesigned and optimized so that the procedure is readily compatible with ESI-LC-MS-MS.

In addition, electrospray ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS) is employed to determine the molecular formula of new metabolite. FT-MS is characterized by its very high mass resolution (Marshall *et al*., 1998; Stensen *et al*., 2000) and unmatched precision of mass determination over a sizeable mass range. FT-ICR MS has been successfully applied in the characterization of some of the most complex natural materials such as natural organic matter (Wu *et al*., 2005; Sannes-Lowery *et al*., 2004). Its high resolution allows virtually any (ionisable) organic compounds to be resolved, even when in a complex mixture present, without a prior chromatographic separation when ion suppression can be avoided. With a 12 -T magnet, a resolving power of

more than 200,000 and a mass accuracy better than 0.2 ppm at m/z 400 can be achieved routinely in full scan mode, which enables exact molecular formula assignment after setting only a few rudimentary chemical constraints. The full spectral sensitivity of ESI-FTICR MS provides highly complementary information with LC/MS.

In this thesis, the existence of dopamine receptor in the *Tetrahymena thermophila* is described. The physiological effects of dopamine are mediated by at least five G protein-coupled receptor subtypes encoded by different genes referred to as D1–D5 (O'Dowd, 1993). Based upon their affinities for classical D1 and D2 ligands, these gene products can be classified into a D1 subfamily encompassing D1 and D5 (D1a and D1b) receptors and a D2 subfamily with the members D2 short, D2 long, D3 short, D3 long, and D4 (Seeman and Van Tol 1994). The pharmacological profiles of these two dopaminergic receptor populations can be discriminated on the basis of their agonist/antagonist specificities (Creese *et al*., 1983). Fluorescent dopaminergic agonist/antagonist ligands play a vital role in the recognition of cellular localization of the two subtypes of dopamine receptors, D1 and D2 (Ariano *et al*., 1989). R(+)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol, the high affinity D1 selective agonist SKF-38393 (Kaiser *et al*., 1982) and 7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine, the high-affinity Dl-specific antagonist SCH 23390 (Iorio *et al*., 1983) were chemically derivatized using the fluorescent compound rhodamine. The modification of these agonists and antagonist ligands has allowed the specific, cellular visualization of the D1 binding site in intact, highly organized regions of cells in a very rapid experimental time frame. The regional localization of receptors labeled by the fluorescent probes is in agreement with previous receptor autoradiography studies.

Biogenic amines are synthesized in some insects such as honey bee (*Apis mellifera*) and fruit fly (*Drosophila melanogaster*). The structure and coupling of their receptors are discussed in the study of Blenau and Baumann (2001). In addition, dopamine and other biogenic amine receptors were proved to exist in the nematode *Caenorhabditis elegans* (Komuniecki *et al*., 2004; Suo *et al*., 2004). However, no previous report could be found describing the biogenic monoamines receptors available in the *Tetrahymena thermophila* although those cells synthesize biogenic monoamines. It is known that G protein–coupled receptors (GPCRs) mediate the activity of dopamine. So far, a variety of protozoa has been screened for the presence of heterotrimeric G proteins and G-protein-coupled receptors. The ciliated protozoa *Stentor coeruleus* and *Chlamydomonas reinhardtii*, have revealed the presence of

heterotrimeric G proteins (Fabczak *et al*., 1993; Korolkov *et al*., 1990) and thus suggesting the existence of dopamine receptor subtypes in such ciliates. In this respect *T. thermophila* is also suggested to translate G protein as well as to contain dopamine receptors.

It is known that many pharmaceutical compounds could finally end up into the aquatic system after their use and intern can affect the microbial ecology of environmental bio-ecosystems. The acute toxicity of 5-fluorouracil and other environmental compounds are discussed in the protozoa and microalgae (*Scenedesmus vacuolatus*). These compounds are continually entering the aquatic environments. 5-fluoruracil is one of the most useful anti-tumour agents and mainly used against cancer for about 40 years. It is usually discharged into hospital waste. This hospital waste generally enters the municipal sewer network without any preliminary treatment and finally contaminates aquatic environments. 5-fluorouracil is extensively used in cancer therapy in combination with other toxic compounds. Recently, the increase in the use of antineoplastic drugs in cancer therapy is considered as an important issue which can affect the environment and in turn affects the microbial ecology. It is expected that the consumption will increase due to the development of the global health care systems. Carcinogenic, mutagenic and reprotoxic (CMR) drugs named as cytostatics are generally enter hospital waste. They are partially transformed or even unchanged via patient's urine and faeces after medical treatment which might therefore contribute the bio-toxicity. In the year 2002, 5-FU was administered to over 2 million patients worldwide and production was estimated to 5000 kg per year worldwide (Malet-Martino *et al*., 2002; Rich *et al*., 2004). Approximately 2-35% of the administered 5-FU is excreted via urine within 24 h (Diasio *et al*., 1989; Schalhorn *et al*., 1992) which indeed enter hospital effluents and may affect normal aquatic life along the food chain. Nowadays many cytostatic drugs have been identified in hospital effluents and their entry and accumulation in the aquatic environment could induce adverse effects in terrestrial or aquatic organisms *(*Hernando *et. al*, 2006*)*. This observation was confirmed in previous studies. Antineoplastic drugs cyclophosphamide, infosfamide, 5-fluorouracil (5-FU), doxorubicin, epirubicin and daunorubicin were identified in hospital waste water samples from the oncogenic department of a hospital at countable concentrations and especially 5-FU was detected at the concentration range of 20-122 µg/L (Mahnik *et al*., 2007; Kümmerer, 2000; Susanne *et al*., 2004; Steger-Hartmann *et al*., 1996).

Therefore, the increasing attention on 5-FU as a potential pollutant may be regarded as a pilot substance for the assessment of environmental contamination originating from hospital

effluents. It is evident that eukaryotic algae are a remarkably diverse and a fascinating group of organisms in the ocean and freshwater. It claims a fundamental nutrient source for all microbial and aquatic life and also plays a role of basic ecological importance as primary producers and is a cornerstone of the food chain *(*Apt *et al*., 1999*)*. This primary producer could potentially accumulate various environmental chemicals e.g. 5-FU. This compound enters into the cell and incorporated into their genetic materials.

The perfluorinated compounds (PFCs) are widespread in the environment. They have emerged as a new class of global environmental pollutants. Among PFCs, perfluorinated sulfonates and perfluorinated carboxylates have attracted much attention in the recent years. These compounds such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in particular, comprises a class of environmentally persistent chemicals (Yamashita *et al*., 2005). One of the most common uses of PFOA is for processing polytetrafluoroethylene (PTFE), most widely recognized under the brand name Teflon by DuPont. Due to their unique physicochemical properties, they have a broad spectrum of applications as surfactants, refrigerants and polymers, and also as components of pharmaceuticals, fire retardants, lubricants, adhesives, paints, cosmetics, agrochemicals and food packaging (Key *et al*., 1997). The telomerization process produces perfluorinated alcohol, which is commonly used in many household surface finishes and indirect contact applications in flexible food packaging. Due to high-energy carbon-fluorine bond, most of PFCs are persistent in the environment and resist hydrolysis, photolysis and biodegradation. They are mostly non-volatile, have high molecular weights, and can repel both water and oils (Bossi *et al*., 2005). However, their persistence, tendency to bioaccumulate, and suspected carcinogenicity has prompted the EU to propose a substantial restriction on marketing and use of PFOS (ES&T Science News, 2006). Despite the widespread use of these compounds, relatively little is known about the fate and effects of PFOS and PFOA, particularly under semi-field conditions. PFOS and related perfluorinated compounds are bio-accumulate in various food chains and have been shown to affect cell-cell communication, membrane transport and process of energy generation, and proxisome proliferation (Upham et al., 1998; Sohlenius *et al*., 1993). Perfluorinated fatty acids alter hepatic lipid metabolism and are potent peroxisome proliferators in rodents. Those compounds demonstrate very low acute biological activity and found EC50 value of 1143-4265 µM in *Vibrio fischeri* and the toxicity increases with longer the perfluorocarbon chain (Mulkiewicz *et al*., 2007). Therefore, in view of their widespread occurrence, extreme persistence, and the fact of their bioaccumulation, the PFOA,

PFOS, 4:2 FTOH, 6:2 FTOH and 8:2 FTOH are further investigated in the *Tetrahymena* species.

In the recent year, nanoparticles (NPs) are increasingly used in the industry and households. Titanium dioxide (TiO_2) is widely used as a white pigment in the production of paints, paper, plastics, sun screen, cosmetic and food colorant. The increasing use of TiO_2 has raised the concern of its toxicity to mammalians. Studies with nano-sized or ultrafine TiO_2 (UF-TiO_2) (<100 nm) particles demonstrated some respiratory toxicity and epithelial inflammation of the lung in rodents and the level of inflammation associated with the particales size, smaller ultratfine TiO_2 caused more effect (Bermudez *et al*., 2002; Bermudez *et al*., 2004; Ferin and Oberdörster, 1985). Ecological studies of TiO_2 is much more limited with few reports of aquatic species like *Daphnia* and rainbow trout (Lovern and Klaper, 2006; Gillian *et al*., 2007). Apart from particle size (one or more dimensions of the order of 100 nm or less) providing a very large surface to volume ratio, their biocompatibility surface properties depend on the charges carried by the particle and its chemical reactivity. Moreover, nanoparticles of TiO_2 and other materials have unique ability to catalyze photo-degradation of various organic compounds (Marci *et al*. 2007). Besides photocatalytic action, nanoparticles could be toxic both for animals, bacteria and algae (Moore, 2006). This research demonstrates the fate and effect of nanoparticles in *Tetrahymena*. Data on biological effects show that NPs can be toxic to bacteria, algae, invertebrates and fish species, as well as mammals. However, much of the ecotoxicological data is limited to species used in regulatory testing and freshwater organism. The possibility of bio-magnification of NP in the food-chain has been mentioned (EPA, 2007), no data are currently available.

The present thesis is organized as: Chapter 2 deals with background of the research, and then after objectives of research. Chapter 3 described the materials and methods of the work as well as calculation behind them. In chapter 4, the results are shown and discussion of the different techniques. A general conclusion of the research is presented in chapter 5 and finally proposed further investigation of this thesis.

2.1 Catecholamines

2.1.1 Biosynthesis of catecholamines

Catecholamines are chemical compounds originated from amino acid tyrosine containing catechol and amine groups. Some of them are biogenic monoamines. They are water-soluble and are 50% bound to plasma proteins. The most abundant and importants catecholamines are adrenaline (epinephrine), noradrenaline (norepinephrine) and dopamine. They are produced via phenylalanine and tyrosine. Tyrosine is created from phenylalanine by hydroxylation by the enzyme phenylalanine hydroxylase. Tyrosine is then sent to catecholamine-secreting neurons. Here, tyrosine is oxidized by tyrosine-3-monooxygenase in the presence of BH4 (6-®-L-erythro-tetrahydrobiopterin) to add another oxygen to form 3,4-dihydroxyphenylanaline (L-DOPA). Dopamine is the first catecholamine to be synthesized by the decarboxylation of L-DOPA. Noradrenaline and adrenaline, in turn, are derived from further modifications of dopamine. The biosynthetic pathway of catecholamines is illustrated in the Fig. 2-1.

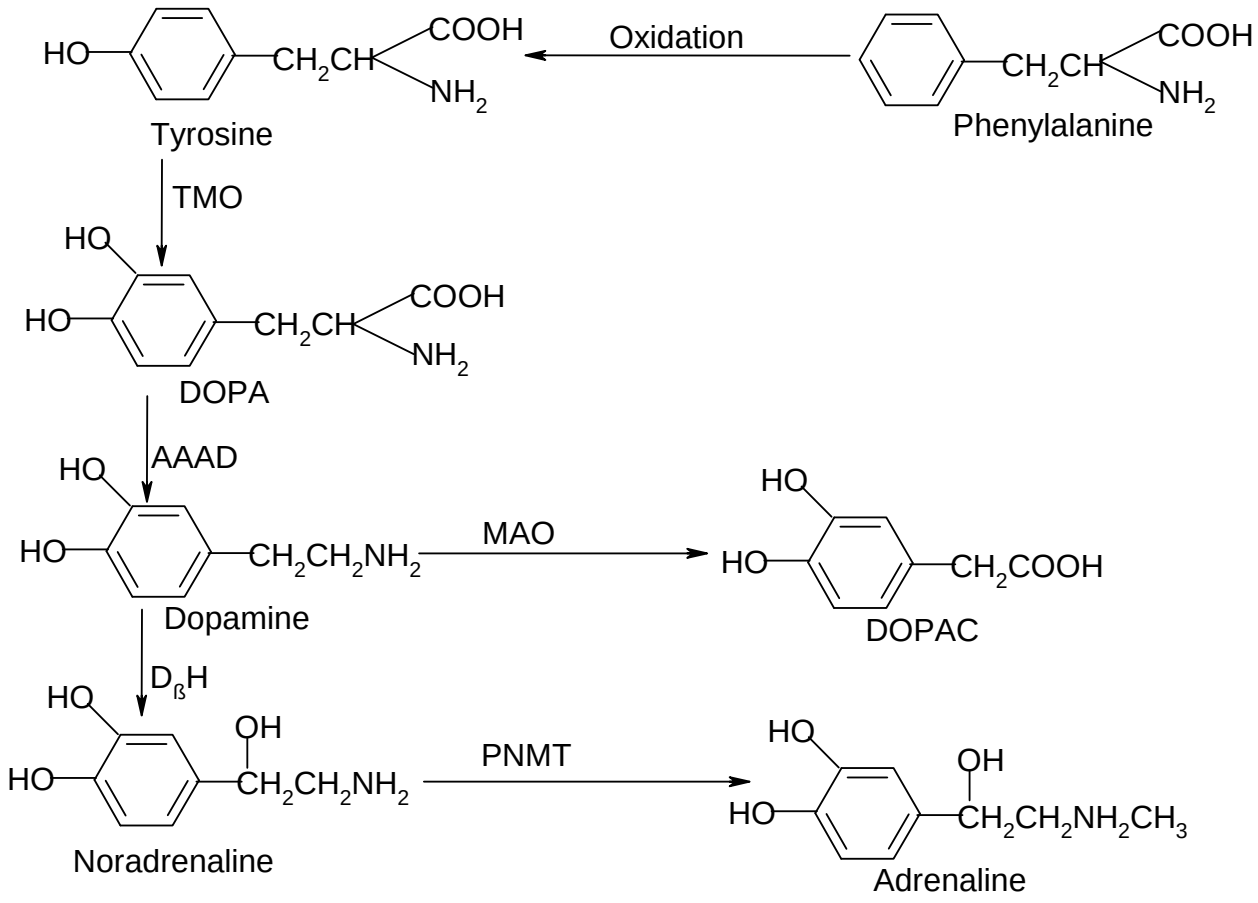


Figure 2-1: Biosynthetic pathway of catecholamine. Tyrosine-3- monooxygenase, L-aromatic amino acid decarboxylase, 3,4-dihydroxyphenylalanine, dopamine ß-hydroxylase, phenylethanolamine-N-methyl-transferase, Monoamine oxidase and 3,4-

dihydroxyphenylacetic acid are abbreviated to TMO, AAAD, DOPA, DßH, PNMT, MAO and DOPAC respectively.

In mammals, catecholamines are produced mainly by the chromaffin cells of the adrenal medulla and the postganglionic fibers of the sympathetic nervous system. Two catecholamines, noradrenaline and dopamine, act as neurotransmitters in the nervous system and as hormones in the blood circulation. Dopamine is largely produced in neuronal cell bodies in two areas of the brainstem: the substantia nigra and the ventral tegmental area. Noradrenaline is a neurotransmitter of the peripheral sympathetic nervous system but is also present in the blood. As definition of hormones, catecholamines are released by the adrenal glands in situations of stress such as psychological stress, elevated sound levels, intense light or low blood sugar levels (Ronald, 1999). Extremely high levels of catecholamines (also known as catecholamine toxicity) can occur in central nervous system trauma due to stimulation and/or damage of nuclei in the brainstem, in particular those nuclei affecting the sympathetic nervous system. In emergency medicine, this occurrence is widely known as *catecholamine dump*. Extremely high levels of catecholamine can also be caused by neuroendocrine tumors in the adrenal medulla, a treatable condition known as pheochromocytoma. Catecholamines cause general physiological changes that prepare the body for physical activity (fight-or-flight response). Some typical effects are increases in heart rate, blood pressure, blood glucose levels, and a general reaction of the sympathetic nervous system. Some drugs, like tolcapone (a central COMT-inhibitor) can raise the levels of all the catecholamines.

2.1.2 Environmental occurrence of catecholamines

Catecholamines are widespread in animals but can also be detected in plants. Dopamine, noradrenaline, adrenaline, and their precursors phenylethylamine and tyramine, as well as other derivatives, have been detected in 44 plant families, including at least 29 species grown for human consumption (Smith, 1977). They are formed in plants from tyrosine, either via tyramine or via 3,4-dihydroxyphenylalanine, L-DOPA (Smith, 1980). Feldman *et al*., 1987 have measured the catecholamines in a number of fruits and vegetables by radioenzymatic assay. However, dopamine is more abundant in plants and measured in higher concentration (5-42 g/g fresh weight (FW)) in the pulp of yellow banana *(Musa acuminata),* red banana *(Musa sapientum* var. baracoa), plantain *(Plantago major),* and fuerte avocado *(Persea americana)*. Dopamine and noradrenaline have been detected in the potato plants (*Solanum*

tuberosum) at the concentration of around 6 μg/g and higher amount was found in the potato leaves (Szopa *et al*., 2001). Cocoa *(Theobromacacao)* bean powder, broccoli *(Brassica oieracea* var. italica), and Brussels sprouts *(B. oleracea* var. gemmifera) contained 1 pg/g, FW, of dopamine. A low dopamine concentration (<l p~g/g FW) was detected in oranges *(Citrus sinensis),* apples *(Malus sylvestris),* tomatoes *(Lycopersicon esculentum),* eggplants *(Solanum meiongena),* spinach *(spinacia oleracea),* beans *(Phaseolus vulgaris),* and peas *(Pisum sativum)* (Feldman *et al*., 1987).

In addition catecholamines act as precursors of benzo[c]phenanthridine alkaloids, which are the active principal ingredients of many medicinal plant extracts. Catecholamines cause the inhibition of indole-3-acetic acid oxidation and enhance ethylene biosynthesis (Kuklin and Conger, 1995). Therefore, the large amounts of catecholamines derived from plants come in contact with soil microcosoms and can affect them. Protozoan species are the most influencing contributor in soil microcosms. Thus the study of catecholamines in protozoan species is of great importance. However, the ciliate protozoan, *Tetrahymena pyriformis* randomly synthesizes biogenic monoamines at countable concentrations. Surprisingly, that cell does not conserve nerve system notwithstanding they produce catecholamines. Some reports have been discussed the identification and detection of all representative members of catecholamines in *Tetrahymena pyriformis* (Goldman *et al*., 1981; Gundersen and Thompson, 1983; Takeda and Sugiyama, 1993) at higher rate in exponential growth phase rather than stationary growth phase.

2.1.3 Adverse effect of catecholamine

Dopamine is an essential neurotransmitter and known neurotoxin that potentially plays an etiologic role in several neurodegenerative diseases. Moreover, due to the oxidation of catecholamine it produces reactive oxygen species (ROS) and quinones; both these species are toxic to the cells (Stokes *et al*., 1999; Cyr *et al*., 2003; Stokes *et al.*, 2000). DA-quinone formation could be catalyzed by various enzymes such as tyrosinase, cyclooxygenase or peroxidase (Hasting, 1995; Napolitano *et al*., 1995; Stokes *et al*., 1996; Wick *et al*., 1977). In consequence DA-quinone contributes to cell damage by macromolecular modification of lipids, proteins, and DNA (Berman *et al*., 1996; Graham *et al*., 1978; Halliwell, 1992; Hastings and Zigmond, 1994; Levay and Bodell, 1993; Sanchez-Ramos *et al*., 1994; Stokes *et al*., 1996). Additionally at higher, pathological concentrations dopamine can cause cell death

both *in vivo* and *in vitro* (Ben-Shachar *et al*., 2004; Stokes *et al*. 2000). Several metabolic pathways of dopamine have been illustrated in the Fig. 2-2 and 2-3. Furthermore, catecholamines are widely used in hospitals, many pharmaceutical products and are entering waste water treatment plants (WWTP), where *Tetrahymena* species are present in analytical solution (AS).

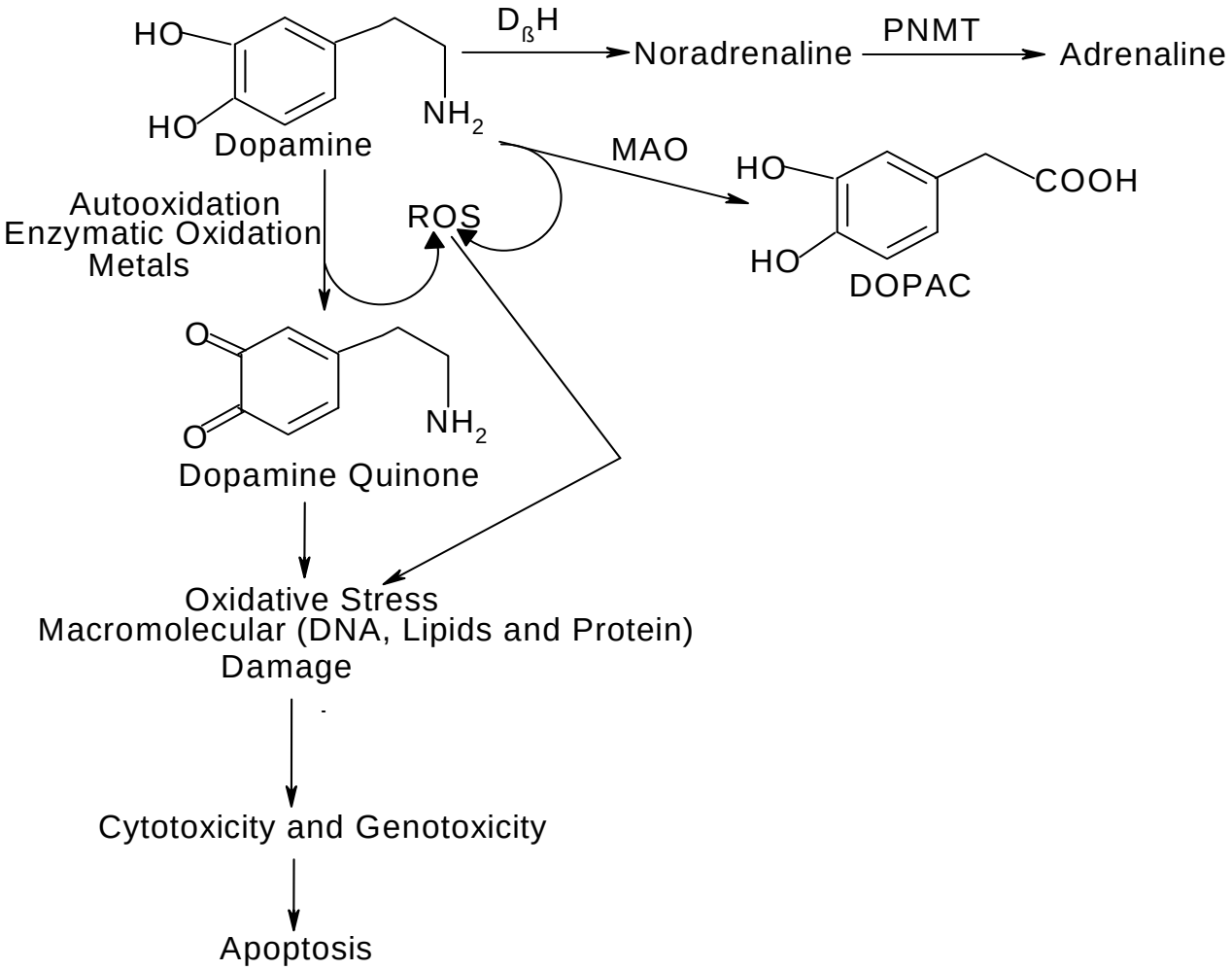


Figure 2-2. Metabolic pathways of dopamine. Dopamine is metabolized in dopaminergic neurons. In noradrenergic cells, dopamine serves as a precursor for the other catecholamines, noradrenaline and adrenaline. Dopamine undergoes oxidation through various mechanisms to a reactive dopamine quinone which has toxic consequences to the cells. Dopamine can also be catabolized by the actions of monoamine oxidase (MAO). Reactive oxygen species, dopamine-ß-hydroxylase, phenylethanolamine-N-methyl-transferase and dihydroxyphenylacetic acid are abbreviated to ROS, DßH, PNMT and DOPAC respectively.

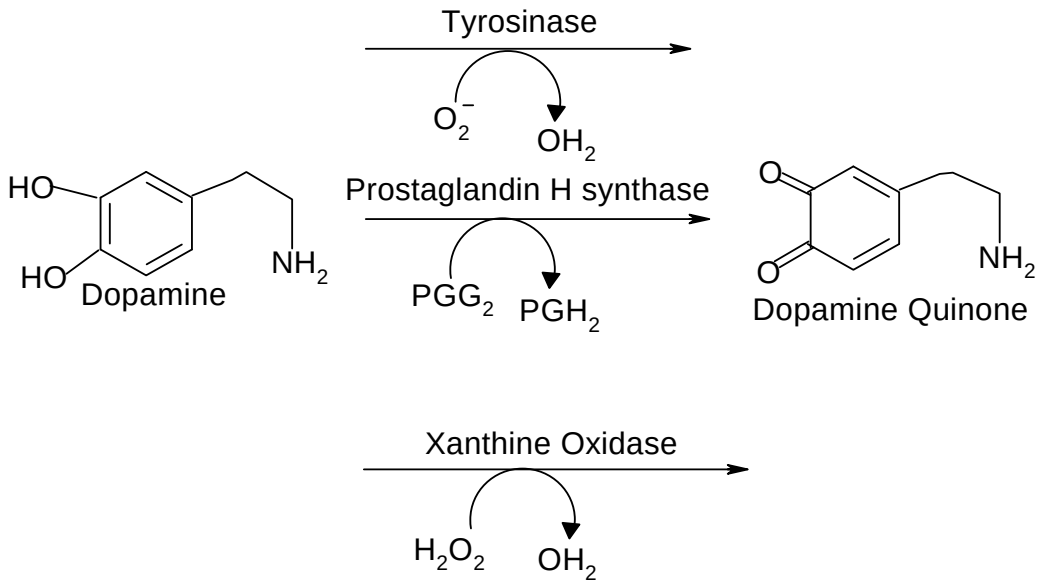


Figure 2-3. Enzymes catalyzed oxidation of dopamine. This figure illustrates different examples of enzymes that cause the canalization of dopamine oxidation.

2.1.4 Catecholamines detection

Catecholamine can be sensitively detected employing HPLC coupled to an electrochemical detector (ED) after solid-phase extraction of cell culture material. The procedure is widely described in the literature (Koller, 1988). Additionally the mass spectrometer (MS) gives structural information about the analytes, which increases the reliability of the identification of analytes. Both tools together provide a highly selective, sensitive for analysis of a wide variety of compounds after an appropriate analytical separation. Electrospray ioniz three ation plays an important role for the analysis and identification of catecholamines (Hirabayashi *et al*., 1995; Rudewicz and Straub, 1986; Kerwin, 1996). Liquid chromatography coupled to mass spectrometry (LC/MS) is a well-accepted technique and a powerful tool for the identification of metabolites and unknown compounds in environmental samples (Hunter *et al*., 2004; Petrovic and Barcelo, 2006; Uebori and Imamura, 2004). Furthermore a sensitive LC–MS–MS analytical technique has been developed for urinary catecholamines after specific sample purification steps (Kushnir, 2002; Wenlin *et al*., 2000) selecting positive ionization mode.

2.1.5 Dopamine receptor in *Tetrahymena*

In both vertebrates and invertebrates, the biogenic monoamine dopamine is implicated in many functions including locomotion, cognition, and development (Mustard *et al*., 2005). The receptor for biogenic monoamines is widely studied in vertebrates in contrast to invertebrates.

Two types of dopamine receptors exist in the cell namely D1 type and D2 type (Seeman and Van Tol, 1994). The D1 dopamine receptor activates cyclic AMP second messenger system, while the D2 dopamine receptor inhibits cAMP signal transduction mechanisms (Enjalbert *et al*., 1986; Lacey *et al*., 1987). The regional distributions of these receptor subtypes have been anatomically distinguished through *in vitro* fluorescent dopaminergic agonist/antagonist ligands (Ariano *et al*., 1989). However, the dopamine receptor mediates their function through G-protein coupled receptor (Girault and Greengard, 2004). Approximately 30% of drugs target to G-protein coupled receptor GPCRs, illustrating their pharmaceutical relevance. The evidence for G proteins is often provided by immunodetection of proteins using antibodies directed against. A variety of protozoa have been examined for the presence of heterotrimeric G proteins and GPCRs (Csaba and Kovacs, 1992; Csaba and Kohidai, 1995).

2.2 5-Fluorouracil toxicity to aquatic organisms

5-Fluorouracil (5-FU, systemic name: 5-Fluor-2,4(1H, 3H)-pyrimidine, MF: $C_4H_3FN_2O_2$, MW: 130.08) is a chemotherapy agent 5-FU (fluorouracil), which has been in use against cancer for about 40 years, acts in several ways, but principally as a thymidylate synthase (TS) inhibitor. Interrupting the action of this enzyme blocks synthesis of the pyrimidine thymidine, which is a nucleotide required for DNA replication. Thymidylate synthase methylates deoxyuridine monophoshate (dUMP) into thymidine monophosphate (dTMP).

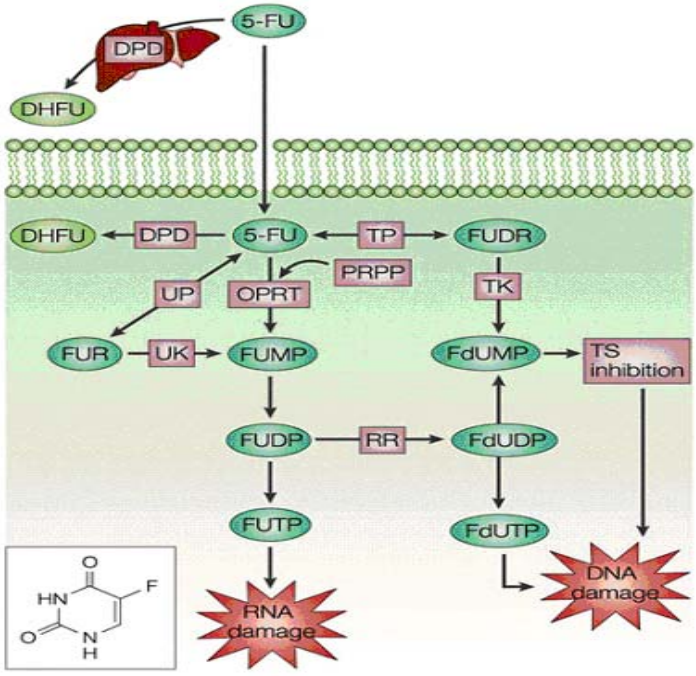


Figure 2-4: Metabolism of 5-Fluorouracil in human.

5-FU is the uracil, one of the four RNA bases found, in which the hydrogen atom in the C-5 position is replaced by fluorine. It transports in the cell, and works as similar mechanisms of uracil. However, in the cell 5-fluorouracil converted to more active metabolites: Fluorodeoxyuridine monophosphate (FdUMP), Fluorodeoxyuridine triphosphate (FdUTP) and Fluorouridine triphosphate (FUTP). These active metabolites disrupt the RNA synthesis by their incorporation instead of uracil or thymidine and inhibit the Thymidylate synthase. In conclusion, 5-fluorouracil transformed inside the cell into different cytotoxic metabolites which are then incorporated into DNA and RNA (Longley *et al*., 2003), finally inducing cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA and RNA. Like many anti-cancer drugs, 5-FU's effects are felt system wide but fall most heavily upon rapidly dividing cells that make heavy use of their nucleotide synthesis machinery, such as cancer. Therefore, 5-Fluoruracil as medicine, particularly used in the hospitals as cytostatic drugs, and after treatment discharged into the sewage water. Because of the high toxic potential, it is absolutely necessary, this substance in the investigation of the effects of drugs in the environment.

5-fluorouracil is not readily biodegradable. The biodegradation was not observed in the closed bottle test (OECD 301 D) nor in the Zahn Wellens test (OECD 302 B), whereas up to 92% degradation was reported in the OECD confirmatory test. Indeed, the degradation rate of 5-FU is directly proportional to the initial concentration (Kümmerer, 1998; Kümmerer and Al-Ahmad, 1997). Its degradation in hospital sewage is decreased, probably as a result of synergistic effect by 5-FU with antibiotics present in hospital sewage (Omra, 2003).

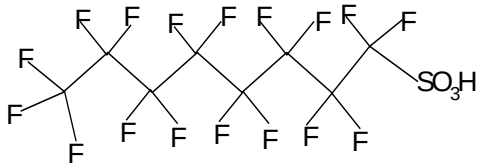
Many pharmaceutical compounds have been detected in the effluents from sewage treatment plants as well as in the aquatic system, e.g., in small creeks and big rivers such as the rivers Rhime, Elbe, Neckar, Danube, Po, and others (Ternes, 1998; Klinger and Brauch, 2000; Zuccato *et al*. 2001) as well as lakes (e.g., Lake Constance, Swiss lakes), in ground water (Heberer *et al*., 1995; Scheytt *et al*., 2000) as well as the North Sea and the Adriatic Sea (Buser and Müller, 1998; Zuccato *et al*., 2001). Emissions from a landfill containing reminders from pharmaceutical production were also reported (Holm *et al*., 1995). Nowadays many cytostatic drugs have been identified in hospital effluents, and the entry and accumulation of these drugs in the aquatic environment could induce adverse effects in terrestrial or aquatic organisms (Hernando *et al*., 2006). 5-FU has been detected in hospital waste water and sewage treatment plants (Mahnik *et al*., 2007; Kümmerer, 2000; Susanne *et al*., 2004). In the environment, 5-

FU is also bio-transformed into toxic fluoroacetate (Arellano *et al.*, 1998) and becomes concentrated to high levels in certain plants after fluoride uptake from soil, water or air (Twigg, 1993) and it affects the growth of bacteria, algae and daphnia (Jorge *et al*., 2007). Several publications have reported that the base analog 5-FU is an inhibitor of RNA metabolism in bacteria (Horowitz *et al*., 1960), animals (Wilkinson *et al*., 1975), and plants (Key, 1966; Fraser, 1975; Francesco *et al*., 1986) and accumulate into r8-S RNA that causes the heterogeneity of ribosomal RNA formation as well as produce faulty proteins in yeast (Mayo *et al*., 1968). Therefore, the toxicity assessment of 5-FU to the aquatic environments might be claimed in prior basis.

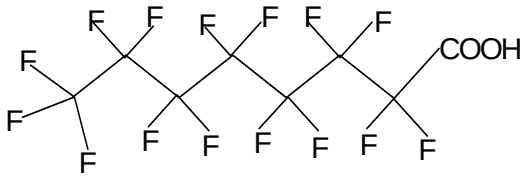
2.3 Effect of perfluorinated compounds to *Tetrahymena*

Perfluorinated compounds (PFCs) have emerged as a new class of global environmental pollutants. They are not only detected in the physical environment, but also in humans and wildlife. However, little is known about the transport and fate of those fluorochemicals in the environment. These contaminants have been found in oceanic waters. Several studies have reported the presence of perfluorinated chemicals in a variety of wildlife species, including freshwater and marine mammals, fish, birds and shellfish (Mulkiewicz *et al*., 2006). Giesy and Kannan, (2001) analyzed PFCs in the samples collected from urbanized areas in North America, especially the Great Lakes region and coastal marine areas and rivers, and Europe and different remote areas. They reported the concentrations of PFOS in animals from relatively more populated and industrialized regions, such as the North American Great Lakes, Baltic Sea, and Mediterranean Sea, were greater than those in animals from remote marine locations. They have found PFOS concentration in the liver sample of 34-3680 ng/g in aquatic mammals, 33-620 ng/g in birds, 7-170 ng/g in fish and 35-700 ng/g in turtles and frogs respectively. Recent studies reported <0.017 to 2,260 μg/l for perfluoroalkane sulfonate (C-6–C-8) and <0.009 to 11.3 μg/l for PFOA in the surface water of a Canadian tributary after an accidental release of fire-fighting foams (Moody *et al*., 2002). Both PFOS and PFOA are detected in water samples from the Tennessee River near a 3M fluorochemical manufacturing site (Hansen *et al*., 2002). Concentrations ranging from a low of approximately 17 ppt (below quantitation limit) upstream of the facility to a high of 144 ppt downstream of the site for PFOS, and a low of 25 ppt to a high of approximately 600 ppt for PFOA were reported. Monitoring of drinking water sources near a production plant in West Virginia indicated the presence of PFOA at below 3 μg/L, substantially below the interim risk screening of 150 μg/L

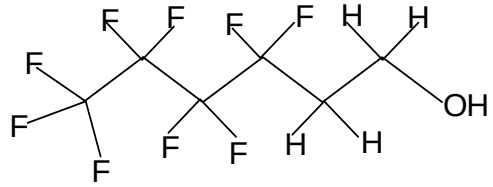
established by the State and US EPA region (West Virginia Department of Environmental Protection, 2002). Surprising, a higher concentration of perfluorinated surfactants upto 4.39 µg/L has been detected in Ruhr River, Germany (Skutlarek *et al*., 2006). The chemical structure of the PFCs illustrated in the Fig. 2-5.



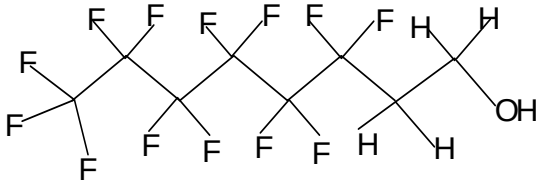
Perfluorooctanesulfonates (PFOS)



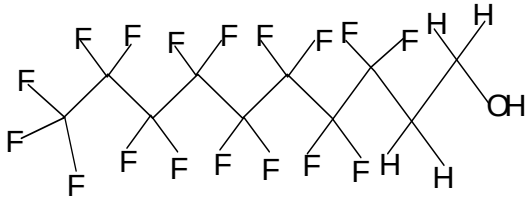
Perfluorooctanoic acid (PFOA)



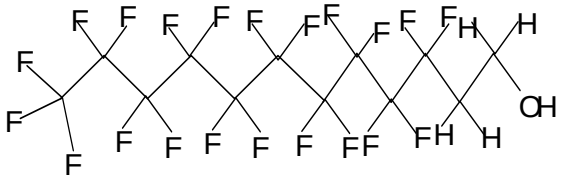
1H,1H,2H,2H-Perfluorohexanol (4:2 PTOH)



1H,1H,2H,2H-Perfluorooctanol (6:2 PTOH)



1H,1H,2H,2H-Perfluorodecanol (8:2 PTOH)



1H,1H,2H,2H-Perfluorododecanol (10:2 PTOH)

Figure 2-5: Chemical structures of PFCs investigated.

The perfluorinated surfactants of fluorotelomer alcohol type (FTOHs) are polyfluorinated compounds typically characterized by even numbered perfluorinated carbons atoms and two non-fluorinated carbons atoms adjacent to a hydroxyl group. Dinglasan *et al*. (2004) reported that FTOHs are probable precursor compounds that may undergo transformation reactions in the environment leading to the formation of these potentially toxic and bioaccumulative PFCAs. FTOHs are typically used as precursor compounds in the production of fluorinated polymers used in paper and carpet treatments and have similar applications as those of PFOS-based products (Kissa, 1994). They are also used in the manufacture of a wide range of products such as paints, adhesives, waxes, polishes, metals, electronics, and caulks (Kissa, 1994). During the years 2000-2002, an estimated 5-106 kg/year of these compounds was produced worldwide, 40% of which was used in North America (Ellis *et al*., 2003).

2.4 Nanoparticles and nonomaterials

2.4.1 Definition of nanoparticles

There are as yet no internationally agreed formal definitions of nanomaterials (NMs) and NPs, but NMs are usually taken to be material with at least one dimension between about 1 nm and 100 nm (Roco, 2003; SCENIHR, 2005; Moore, 2006). These could be materials such as nanofilms (one dimension), nanowires and nanotubes (two dimensions), or nanoparticles (three dimensions). However this is a somewhat arbitrary definition, and for ecotoxicology, we should also consider NMs with a distribution of particle sizes around the nanoscale that may include some primary particles larger than 100 nm; or larger aggregates of nanoparticles (NPs) of a few hundred nanometers (Handy and Shaw, 2007; Handy *et al*., 2008). A precise size threshold in this definition is not intended, since it is the novel toxic effects due to small size that are of interest. However, a pragmatic solution might be to consider materials with a primary dimension of <0.5 μm to differentiate nanoscale from micrometer scale. Whatever pragmatic decision is made about size thresholds to define NPs, some flexibility is needed. For example it could be argued that a single solid particle of 0.5 μm diameter might not be different from the behaviour of a slightly larger particle in the 1–2 μm range. However aggregates with an overall dimension in the μm range, but made of primary particles of <100 nm would be regarded as a NM. Clearly for NPs, the primary particle size should be considered (e.g., the diameter of a single particle). In addition, the sizes of aggregates of NPs, which can be several hundred nanometers or more, and the distribution of particle sizes present in the material also need to be considered. In mammalian respiratory toxicology sizes of particulate matter (PM) have been traditionally defined as coarse particles (diameter between 10 and 2.5 μm, $PM_{10–2.5}$), fine particles (2.5 μm or less, $PM_{2.5}$), or ultrafine particles (<0.1 μm, $PM_{0.1}$). Nanoparticles can therefore also be regarded as ultrafine particles, and emerging ecotoxicological data might be compared with some of the ultrafine particle exposures performed on rodents (Handy and Shaw, 2007)

2.4.2 Occurrence of nanoparticles

Nanoparticles (NPs) are used in many commercial products and frequently enter to the aquatic environments. Therefore, their mobility, bioavailability, and assessment of toxicity to aquatic microcosms are of important concern. Nanoparticles are not a human invention and have existed naturally from the beginning of the earth's history, and natural sources can be

found in volcanic dust, most natural waters, soils and sediments (Murr *et al*. 2004; Verma *et al*., 2002). There are several mechanisms that create NPs in the environment and these can be either geological or biological. Geological mechanisms include physicochemical weathering, authigenesis/neoformation (e.g. in soil), and volcanic activity. These processes typically produce inorganic NPs. Biological mechanisms typically produce organic NPs, although some organisms can produce mineral granules in cells. Natural occurrences of nonmaterial are summarized in the Table 2-1.

Table 2-1: Natural occurrence of nanoparticles

Location of NPs	Particle types and ecotoxicological potential	Authors
Volcanic ash	Volcanic ash from the Montserrat eruption contain Cristobalite (crystalline silica) causes lung inflammation and lymph node granuloma in laboratory rats	Lee and Richards, 2004
Volcanic dust	Bismuth oxide nanoparticles were found in the stratosphere in 1985, and the presence of these materials was linked to volcanic eruptions in the 1980s	Rietmeijer and Mackinnon, 1997
Ocean surface microlayer (SML)	It contains colloids, sub-micron components of phytoplankton, and carbon particles. There are concerns that organic pollutants present in the SML may be adsorbed to colloids and other nano-scale material in the SML.	El Nemr and Abd-Allah, 2003; Obernosterer *et al*. 2005
Soil	A complex matrix containing mineral particles, colloids in pore water, and there are concerns about adsorption and binding of pollutants within the matrix	Reid *et al*., 2000
Freshwater	It contains very complex colloid material which includes inorganic minerals and organic matter such as humic substances. Concerns exist over the accumulation and transportation of NPs in the colloid fraction	Lead and Wilkinson, 2006
Other natural waters	Nanoparticles were found in many types of natural water including the oceans, surface waters, groundwater, atmospheric water, and even treated drinking water. These include a wide variety of nanoscale mineral particles, and demonstrates the ubiquitous nature of nanoparticles in the natural environment	Wigginton *et al*., 2007
Ice cores	Surprisingly, carbon nanotubes, carbon fullerenes and silicon dioxide nanocrystals have been found in 10,000 year old ice cores	Murr *et al*., 2004
Historic sediments	Cretaceous-Tertiary (K-T) boundary layer at Gubbio, Italy showed iron particles (hematite) and silicates. The average particle size of the hematite was 16–27 nm. There is speculation that meteorite impacts could alter NP formation in sediments at the K-T boundary	Verma *et al*., 2002

Furthermore, NPs also come from point sources such as production facilities, landfills or waste water treatment plants or from nonpoint sources such as wear from materials containing

NPs. Accidental release during production or transport is also possible. In addition to the un-intentional release there are also NPs released intentionally into the environment. The particles are released directly into water/soil or the atmosphere; they all end up in soil or water, either directly or indirectly for instance, via sewage treatment plants, waste handling or aerial deposition. The important processes and pathways of NPs in the environment are depicted in Fig. 2-6. The globally production of NPs are estimated about one billion metric tons per year (Kellogg and Griffin, 2006), and even a fraction of this as ultrafine particles, this would be million of tons of natural nanoparticles.

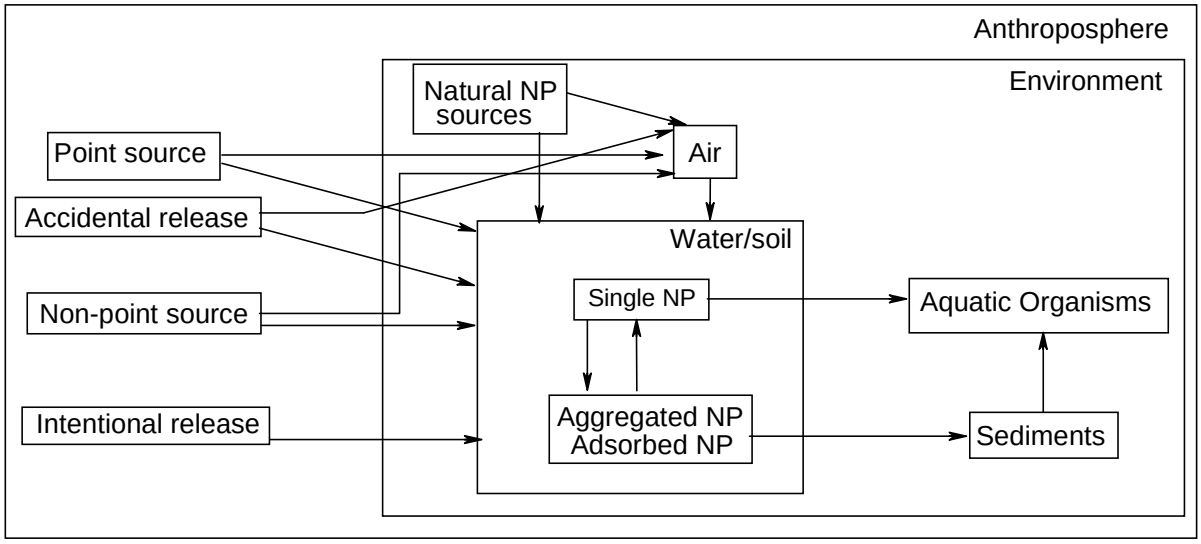


Figure 2-6: Pathway of NP from the anthroposphere in the environment, reaction in the environment and exposure to aquatic organisms.

2.4.3 Awareness of nanoparticles to ecotoxicologist

The environment does contain many natural particles at the nm scale such as colloids in freshwater (colloids are materials in the size range 1 μm to 1 nm, Lead and Wilkinson, 2006). It could be argued that these materials have been in the environment for millions of years and organisms must be adapted to living in the presence of these natural substances. There are also concerns that anthropogenic activity has been incidentally generating nano-scale pollutants such as air-borne particles from car exhausts or nanoparticles generated from the erosion of materials such as car tyres for a long time (Handy and Shaw, 2007). Even so, we still have much to learn about the fate and behaviour of natural colloids and their interactions with pollutants (Lead and Wilkinson, 2006). Manufactured NPs might represent a special case, since they may be designed to have particular surface properties and (surface) chemistries that are less likely to be found in natural particles. They might therefore present enhanced or novel physico-chemical or toxicological properties in comparison to natural NPs.

Manufactured NPs (and natural NPs) often exhibit special physico-chemical properties and reactivities due to their small size and homogeneous composition, structure or surface characteristics, which are not present at the larger scale. So, for example, carbon fullerene NPs (C_{60} particles), may have a different toxicity compared to fine (μm sized) graphite particles, even though both particles are made of carbon (Barlow *et al*., 2005). In particular NPs possess a much higher specific surface area (SSA) than their larger counterparts of the same material, and the proportion of atoms on the surface versus the interior of the particle is also much larger for NPs. Together, these factors can give rise to a higher surface reactivity (e.g., adsorption and/or catalytic properties) for the same mass of material. This gives rise to the suggestion that SSA (e.g., m^2/g of material) rather than mass concentration (e.g., mg/L) might sometimes be more important to the toxicity of NPs, and is perhaps a better way to describe the dose–effect of nanomaterials when surface reactivity is a key characteristic (Oberdörster *et al*., 2007). Of course, the total surface area available will be a function of SSA multiplied by the particle mass concentration, so both are likely to be important in exposure. Also, the importance of shape and particle surface area in the uptake of NPs across the cell membranes of many organisms remains to be established in ecotoxicology.

In addition, the variety of physical structures of NMs, (e.g., different crystal structures of the same material), and the potential for these structures to contain more than one substance (e.g., Ag–Ti composites as antibacterial coatings), or to be manufactured with multiple types of surface ligands, creates a new challenge for ecotoxicity testing.

The use of the waterflea, *Daphnia magna*, as a standard ecotoxicology test species is well known, and it is perhaps no surprise that early studies have focused on this organism. The C_{60} fullerenes and TiO_2 nanoparticles cause the mortality of D. magna and mortality increase with concentration (Lovern and Klaper, 2006). Oberdörster *et al*. (2006) have also investigated chronic effects in *D. magna* and other invertebrates. *D. magna* were exposed to up to 5 mg/L C_{60} fullerenes over 21 days. At the highest C_{60} concentration (5.0 mg/L), mortality of 40% was observed, along with fewer offspring and a delay in moulting. In the same study, exposure of the freshwater invertebrate, *Hyallela azteca*, to 7 mg/L C_{60} fullerenes for 96 h was reported to have no effect on mobility, moulting or feeding behaviour (Oberdörster *et al*., 2006). More recently, several studies have noted behavioural effects of NPs in *D. magna* (Lovern *et al*., 2007; Roberts *et al*., 2007). Lovern *et al*., (2007) noted changes in locomotor behaviour (hopping frequency and appendage movement) and Roberts *et al*., (2007) found that *D. magna* use the organic lysophosphatidylcholine coating on SWCNTs as a food source.

C_{60} nanoparticle interferes with the embryonic development of Zebrafish (Zhu *et al*., 2007). Published reports on the ecotoxicity of manufactured NPs to soil invertebrates and other terrestrial invertebrates appear to be lacking, although aware of preliminary studies with earthworms (Scott-Fordsmand *et al*., 2007).

3.1 Experimental model organism

There are two types of model aquatic microorganism used for this thesis. One is ciliated protozoan, *Tetrahymena spec*. and another is the green micro-algae *Scenedesmus sp.*

3.1.1 Ciliated protozoan, *Tetrahymena*

Tetrahymena are non-pathogenic free-living ciliate protozoa. They are common in fresh-water. *Tetrahymena* possesses hundreds of cilia and exhibits striking nuclear dimorphism: two types of cell nuclei exist in a single cell at the same time and carry out different functions with distinct cytological and biochemical properties. They can be easily cultured in a large quantity in the laboratory. Also they can grow very rapidly to high density in a variety of media and condition due to small generation time. It is an eukaryotic organism for molecular and cellular biology (Collins and Gorovsky, 2005) animal free testing obligations. In recent years, *Tetrahymena* has been a great source for biochemical analysis of important enzymatic activities and environmental toxic chemicals. A functional diversity encoded in the genome and the presence of some pathways missing from other model organisms makes *Tetrahymena* an ideal model for functional genomic studies to address biological, biomedical, and biotechnological questions of fundamental importance.

The test was conducted with two different species of *Tetrahymena*. One is *Tetrahymena pyriformis;* strain GL which was received from the Institute of Ecotoxicology and Biochemistry**,** Free University of Berlin, Germany. The other species is *Tetrahymena thermophila* which was purchased from Tetrahymena Stock Center, C5 155 Veterinary Medical Center, Cornell University, Ithaca, New York 14853. *Tetrahymena pyriformis* was considered for toxicity assay of all compounds in addition with investigations about catecholamine homeostasis. However, *Tetrahymena thermophila* has advantages for the study of molecular biology instead of *Tetrahymena pyriformis*. The life cycle of *T. thermophila* allows to use them as conventional tool of genetic analysis due to the development of molecular genetic tools for sequence-enabled experimental analysis of gene function (Asai and Forney, 2000; Turkewitz *et al*., 2002). In addition, although it is unicellular, it possesses many core processes conserved across a wide diversity of eukaryotes (including humans) that are not found in other single-cell model systems (e.g., the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*). They exert massive genetic information and currently 140

Mbp genome sequences are known for it (Stover *et al*., 2006). The cells are illustrated in the Fig. 3-1. However, both species are quite similar in shape and size.

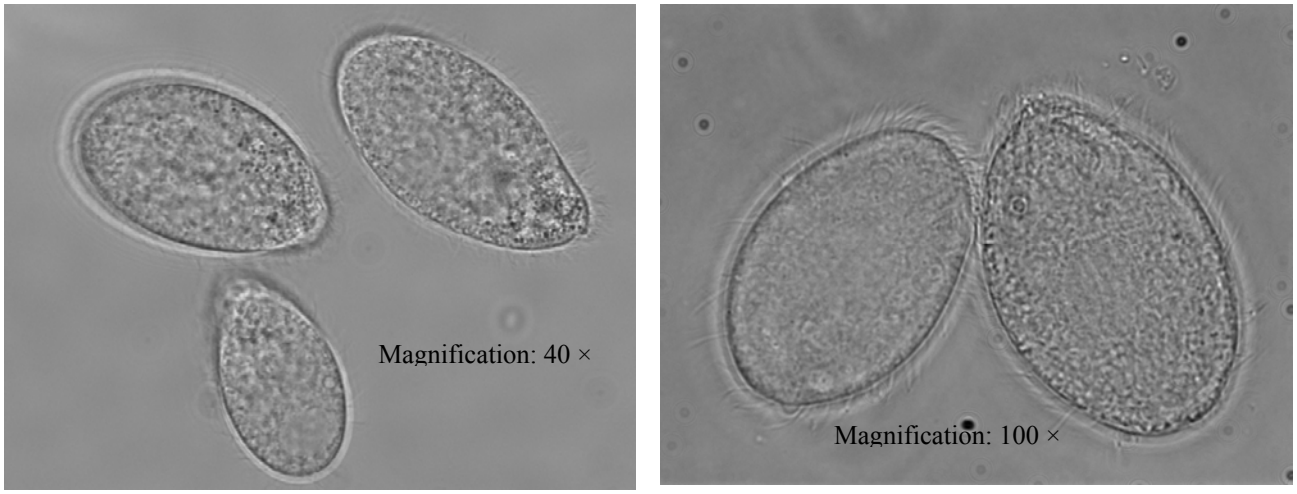


Figure 3-1: *Tetrahymena thermophila* SB210

Scientific classification of *Tetrahymena*:
Domain: Eukaryota; Phylum: Ciliophora; Class: Oligohymenophorea; Order: Hymenostomatida; Family: Tetrahymenidae; Genus: *Tetrahymena*; Species: *T. thermophila*

3.1.2 Green microalgae, *Scenedesmus vacuolatus*

Green Microalgae are typically found in freshwater and marine systems. They are often referred to as microphyte. Microalgae such as microphytes constitute the basic foodstuff for numerous aquaculture species. Therefore, they contribute a bioaccumulation factor in the aquatic environment. However, in this study *Scenedesmus vacuolatus* (old name *Chlorella fusca*) was considered for toxicity assay with of 5-fluoriuracil. The organism was obtained from the Institute of Plant Physiology, University of Göttingen, Germany.

Scenedesmus is an unicellular species which exist individually (Fig. 3-2). It is planktonic mainly in eutrophic freshwater ponds and lakes, rarely in brackish water; reported world-wide in all climates. It has relatively uniform physiology and biochemistry. *Scenedesmus* is highly polymorphic in culture with variation induced by various culture conditions. Therefore this microorganism could be strangely used as model organism for the study of environmental chemicals.

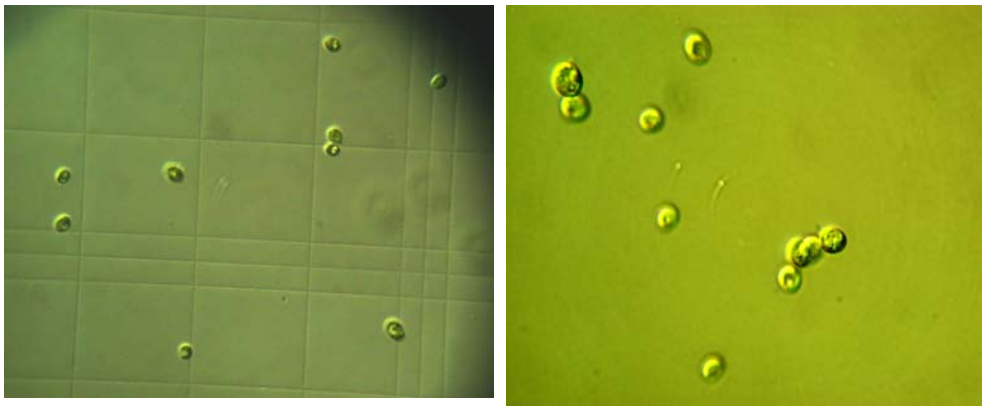


Figure 3-2: *Scenedesmus vacuolatus* (magnification:

Scientific classification of Scenedesmus:
Domain: Eukaryota; Kingdom: Plantae; Phylum: Chlorophyta; Class: Chlorophyceae; Order: Chlorococcales; Family: Scenedesmaceae; Genus: *Scenedesmus;* Species*: Scenedesmus vacuolatus.*

3.2 Preparation of the cell culture medium

3.2.1 Culture medium of *Tetrahymena*

Tetrahymena cell was cultivated in proteose peptone yeast extract medium. The medium was prepared with the combination of proteose peptone and yeast extract with the addition of glucose and trace elements (Plesner *et al*., 1964). To avoid pH-shift during the cell culture or due to the addition of test chemicals, the PPY-medium was pH stabilized by a biological buffer. PPY medium was prepared in Millipore water (Milli-Q® Ultrapure Water Purification Systems, Concord Road Billerica, MA, 01821, USA) and its composition is as follows:

Organic contents:

a. 3.5 g/L Proteose peptone (Merck Bioscience Ltd., U.K)
b. 0.7 g/L Yeast extract (Fluka Biochemika, Switzerland)
c. 3.5 g/L Glucose (Fluka Biochemika, Switzerland)

Salts/trace elements, added separately to the culture medium (all salts are purchased from Sigma-Aldrich Chemie GmbH Laboratory, Germany)

50 mg/L	$CaCl_2.2H_2O$	added as 100× concentrated
5 mg/L	$CuCl_2.2H_2O$	Salt solution 1
1.25 mg/L	$FeCl_3.6H_2O$	

100 mg/L	$MgSO_4.7H_2O$	added as 100× concentrated
25 mg/L	$Fe(NH_4)_2(SO4)_2.6H_2O$	Salt solution 2
0.5 mg/L	$MnCl_2.4H_2O$	
0.05 mg/L	$ZnCl_2$	

Biological pH-Buffer:

2.093 g/L, 3-(N-Morpholino)propanesulfonic acid (Mops) buffer

pH 7.2, NaOH.

This medium was used as nutrient supply for the cultivation of experimental stock, pre-culturing of organism and conducted exposure experiments.

3.2.2 Culture medium of microalgae

The isolates of the unicellular green microalgae, *Scenedesmus vacuolatus* are cultivated anexically in an inorganic medium. All the inorganic salts were purchased from Sigma-Aldrich Chemie GmbH Laboratory, Germany. The algae medium was prepared by taking 20 mL solution of NaNO3 (24.8 g/500 mL), $K_2HPO_4.3H_2O$ (2.55 g/500 mL), $MgSO_4.7H_2O$ (3.75 g/500 mL), $CaCl_2.2H_2O$ (1.8 g/500 mL), Na_2CO_3 (2.9 g/500 mL), $Na_2EDTA.2H_2O$ (0.5 g/500 mL), FeIII Citrate (0.15 g/500 mL) and $C_6H_8O_7.H_2O$-Citric acid (0.15 g/500 mL) and 2 mL trace elements (15 g/L) into 2 L conical flask. Afterwards, distilled water was filled into the flask up to the mark. This inorganic medium was subjected to sterilization for 30 min. Due to the sterilization of medium a precipitate is formed which was dissolved by continuous flowing with 3% CO_2 in air. During CO_2 flow the pH in the medium is equilibrated approximately 8.

3.3 Cultivation of test organisms

3.3.1 *Tetrahymena sp.*

3.3.1.1 Long term stock culture

Long term culture of the cells was managed in glass stock vials with screw top. single chick-pea and 10 mL Millipore water were taken together into glass stock vials and kept overnight at 4^{o}C. Afterwards, the vials are sterilized for 20 min and stored one day at room temperature. 100 µL exponential growing cell cultures were added into the glass vial at sterile condition and preserved for several months.

3.3.1.2 Experimental stock cultures

The experimental stock culture was prepared for every week from long term stock culture. This culture was reared in PPY-medium and serve as starter culture for the preparation of

logarithmically growing pre-culture. 200-300 µL cell aliquot was transferred from the vial by piercing of septum with a sterile syringe and added into 40 mL PPY medium in a 300 mL Erlenmeyer flask with fine porous silicon gum head. The porous cap of the flask allows an adequate oxygen supply. Temperature of the culture flask was maintained to 28^{o}C as same for chemical toxicity studies too. For routine purposes it was prepared on Friday and incubated over weekend to have experimental stock throughout the following week.

3.3.1.3 Experimental pre-cultures

Exponential growing pre-cultures are basic required for testing catecholamines and other compounds. Pre-cultures were set up one day prior to test transferring cells from experimental stock cultures. They were also cultivated under the same conditions as for test. 300-500 µL aliquots of the experimental stock culture was taken by a pipette under sterile conditions and transferred into a 300 mL Erlenmeyer conical flask with cap containing 40 mL PPY-medium. Afterwards, the culture was incubated for 22-24 hours at 28^{o}C to obtain suitable exponential dividing cell. The cell growth was checked with spectrophotometer (SLT-spectra, Salzburg, Austria) by measuring optical density (OD). The optical density (λ = 550 nm) for a one day old culture usually shows between 0.11-0.30 which holds the cell concentration to 1-4×10^{5} cells/mL.

3.3.2 *Scenedesmus sp.*

The culture of *Scenedesmus vacuolatus* was prepared by seeding inoculums from the slant agar into 2 L Erlenmeyer flask by a loop under sterile condition. The flask is then placed in the culturing apparatus which maintains continuous shaking and illumination. During algae cultivation, the cell suspension was facilitated by continuous transfer of 3% CO_2 in air. Medium was continuously stirred with magnet bar. The temperature of the culture environment was maintained between 21 and 25^{o}C, controlled at ±2^{o}C. Prior to the experiment, algae cells were acclimatized in the fresh medium with twice successive reseeding. However, the acclimatization of cell was assumed to be gain of capacity to grow exponentially which is essential for testing chemicals.

3.4 Catecholamine haemostasis in *Tetrahymena*

3.4.1 Stock solution of catecholamine

The five representative catecholamines dopamine (DA), 3,4-dihydroxyphenylalanine (L-DOPA), 3,4-dihydroxyphenyl acetic acid (DOPAC), noradrenaline (NA) and adrenaline (A) are tested against *Tetrahymena*. The test compounds dopamine-hydrochloride, L-DOPA, DOPAC and DL-adrenaline were purchased from Sigma-Aldrich Chemie GmbH Laboratory, Germany. DL-noradrenaline hydrochloride was purchased from Fluka Biochemika, Switzerland. The compounds were dissolved in Millipore water which prepared by Milli-Q® Ultrapure Water Purification Systems (290 Concord Road Billerica, MA, 01821, USA). Concentrations of stock solutions were prepared to 80 g/L. The stock solution was diluted 500 folds in PPY-medium to achieve the highest desire concentration in the 96 well plates.

3.4.2 Determination of toxicity of catecholamines in *Tetrahymena*

The toxicity of all representative catecholamines (L-DOPA, DA, NA, A and DOPAC) were determined by considering cell growth inhibition of *Tetrahymena pyriformis*. At the beginning of the experiments the cell concentration of pre-culture was adjusted to 5×10^4 cells/mL with the addition of PPY-medium. Cell was counted by Neubauer cell counter (Brand Wertheim, Germany). Stock solutions of the five different catecholamines were also directly diluted in PPY-medium. 200 µL test chemicals and 50 µL log-phase *Tetrahymena* culture on the top of chemicals are transferred into 96-well plates (Gereiner bio-one, Germany) to have a convenient testing volume of 250 µl/well. Inoculum was added on the top of the chemicals. The initial cell concentration in the well plates ultimately reached to 1×10^4 cells/mL. Well plates were prepared under sterile condition (Heraeus LaminAir® HBB 2448 S). Highest concentration of tests compounds in the well plate was 80 ppm which decreased to 0.078 ppm by two fold dilution series (eg. 80 ppm, 40 ppm, 20 ppm etc.). Finally well plates were shaken gently to achieve homogeneous distribution of cells throughout the test volume. Afterwards, the test plates were placed into an incubator (Kelvitron®t, Heraeus, Germany) at 28°C. Cell growth was monitored for 24 h (T_1) and 48 h (T_2) exposure respectively. In this experiment, growth of the cell was measured by taking optical density (OD) with a spectrophotometer at 550 nm. OD is directly proportional to the number of *Tetrahymena* cells and the percentage of inhibition of the cell growth rate for each treatment

replicate (n=4) was determined in comparison to control according to the equations number 1 and 2.

Average specific growth rate, $\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i}$ (day $^{-1}$) ……..…….1

Where,

μ_{i-j} is the average specific growth rate from time i to j;

X_i is the OD at beginning time (zero days);

X_j is the OD at time first day or second day exposure.

For each treatment group and control group mean value of average specific growth rate is used for calculation of growth inhibition as follows.

% of Cell growth rate inhibition, $\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100$ …….. 2

Where,

$\%I_r$ is the percent of cell growth inhibition in average specific growth rate;

μ_C is the mean value for average specific growth rate (μ) in the control group;

μ_T is the mean average specific growth rate for the treatment group.

The effective concentration (EC_{10} and EC_{20}) of catecholamines are derived graphically from a plot of percentage of inhibition against the logarithm value of the concentration of the substance. EC values are read from the curve at intercept of the line with a parallel drawn to the abscissa at 10% and 20% respectively.

3.4.3 Quantitative analysis of catecholamine employing HPLC-ED

3.4.3.1 Instrumentation and reagents

The quantitative estimation of noradrenaline, adrenaline and dopamine from *Tetrahymena pyriformis* cell and biological control materials was perform employing high-performance liquid chromatography (HPLC)-electrochemical detection (ED).

The HPLC system consisted of a GP40 gradient pump, an ED40 electrochemical detector, AS50 autosampler (all were purchased from Dionex Corporation, 1228 Titan Way,

Sunnyvale, CA 94085, USA). AS50 auto-sampler was equipped with a 100 µl loop. An analytical column "Clin Rep® Catecholamines in Plasma" was filled with reversed-phase C18 material (Recipe, Munich, Germany) is kept in a column oven which maintains constantly 25°C temperature. A PEEK pre-column filter with a 3 µm Peek/PTFE frit (Recipe, Munich, Germany) is placed between the sample injection valve and the HPLC column to protect the column from particles originating from the sample and pump/valve seal-wear.

The ED40 amperometric detector (Dionex) was used to control detector voltage and record the current. A glassy carbon electrode (Dionex) was served as the working electrode, and is used together with a Ag/AgCl reference electrode and a stainless steel auxiliary block as the counter electrode (all from Dionex, Germany). That working electrode is manually set at a potential of +700 mV versus Ag/AgCl reference electrode. The total run time of analysis is fixed for 15 min (with re-equilibration of the column) at a flow rate of 1 ml/min. Current flow is maintained below 10 nA by polishing the working electrode to have a more sensitive detection of catecholamines.

The mobile phase was purchased from the Clin Rep® commercial HPLC kit which is used in a completely isocratic mode for the determination of catecholamines. The sample preparation columns including elution vials containing aluminium oxide suspensions for the adsorption of catecholamines and other necessary solutions (buffers for adsorbing, washing and etc.) were purchased from the 'Clin Rep® Catecholamines in Plasma kit'. The internal standard solution (3,4 dihydroxybenzylamine, DHBA) and standard solution for external calibration containing noradrenaline, adrenaline, DHBA and dopamine were also collected from the Clin Rep® commercial HPLC kit.

Blood plasma samples (biological control materials, level I+II, Recipe, Munich, Germany) are pipetted together with 50 µl (=500 pg) internal standard DHBA into the sample preparation columns and extracted according to the instruction manual of the manufacturer (Thomas, 1998). Samples of *Tetrahymena pyriformis* were also extracted and measured under identical conditions as described for blood plasma samples.

The analytical precision, accuracy and the lower limits of determination were determined from biological control materials. The recovery rates for the catecholamines in the control

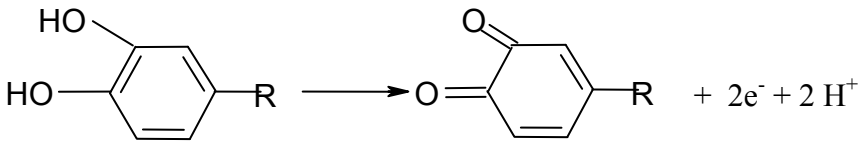
materials (blood plasma) and *Tetrahymena* samples are above 80%. The detection limit for all catecholamines in HPLC-ED is below 5 ng/L.

3.4.3.2 Mechanism, description and calculation of catecholamine concentration

Here, the catecholamines are determined by electrochemical detection, a method of high sensitivity, specificity and selectivity. The specificity and selectivity of the electrochemical detection is due to the fact that only those kind of compounds can be detected, which may be easily oxidized or reduced on the surface of a working electrode. The electrochemical turn over of a substance on the working electrode leads to a loss or to an uptake of electrons, resulting from oxidation or reduction. The arising current is proportional to the concentration of the substance flowing through the detector cell. The current is detected by a measuring device, amplified and displayed as a chromatogram. Molecules which may be easily oxidized always have certain functional groups, such as:

- Hydroxyl groups on the benzene ring
- Amino groups on the benzene ring
- Thiol groups
- Heterocyclic N- and S-atoms

Thus, the detection of the catecholamines by oxidation to the corresponding ions may be represented as follows:



Prior to the detection step, each sample has to be prepared in a short sample preparation. Each sample was labelled according to the test. The sample preparation column contains a defined amount of activated aluminum oxide (22 mg), which was suspended in a 2M Tris-HCl buffer solution. pH value (secrete by manufactures) of this buffer is adjusted to enable a maximal and selective adsorption of the catecholamines. Desired volume of cell culture (200 µL) is spiked with an internal standard (50 µL). These were placed into the sample preparation column. The columns are vortexed for 10 min. As a consequence, the catecholamines are adsorbed at the aluminum oxide and are isolated from the sample matrix (Fig.3-3). The cell

culture is now free of catecholamines and removed by vacuum station. Interfering substances, being co-adsorbed at the aluminum oxide, were removed by three consecutive washing steps. Afterwards 200 µl catecholamines were eluted on stabilizing conditions from the sample preparation column. Finally 50 µl of elute was injected into the HPLC system.

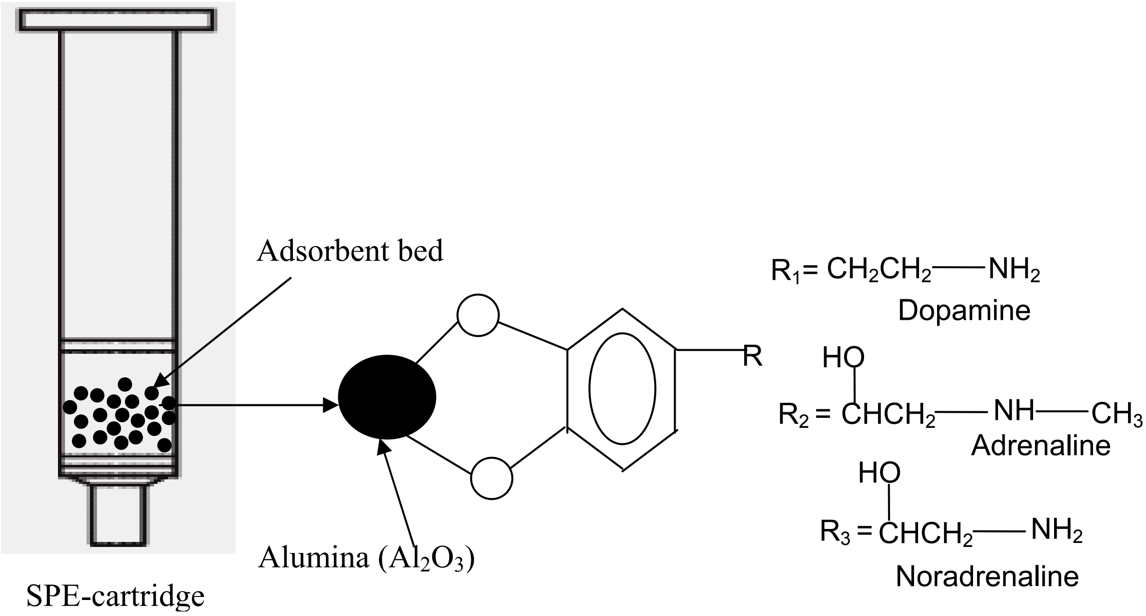


Figure 3-3: Mechanism of catecholamine extraction.

A special reverse-phase column was used for the chromatographic separation. The analytes are measured by an electrochemical detector and, using the internal standard method, and evaluated quantitatively. It is ensured that each chromatogram corresponds to the appropriate sample and marks the peak of chromatograms. The standard chromatogram of a catecholamine mixture was compared with the chromatogram of analyte present in the test sample. Each chromatogram has been compared with that of the standard. The peaks of analyte in the standard mixture were checked whether they represented and correlated to the analyte in the sample with respect to the retention time. Concentration of catecholamines was calculated by the recovery of internal standard (IS) is as follows formulas:
Calculation of the recovery (REC) of internal standard,

$$REC = \frac{\text{Area}_{\text{IS}}\text{, sample} \times 10 \times 200}{\text{Area}_{\text{IS}}\text{, standard} \times 4 \times 500} \quad \ldots\ldots\ldots \quad 3$$

Where,
Sample amount = 4
Amount of IS in 50 µL = 500 pg
Elution volume of sample = 200 µL
Concentration of IS = 10 pg/µL

$$\text{Concentration of analyte} = \frac{\text{Area}_{\text{analyte, sample}} \times \text{Conc.}_{\text{analyte, std.}} \times 200}{\text{Area}_{\text{analyte, standard}} \times \text{Vol.}_{\text{sample}} \times \text{REC}} \quad \text{..... 4}$$

Where,
Elution volume of sample = 200 µL
Conc. $_{analyte}$, std. = Concentration (pg/µL) of analyte in standard
Vol. $_{sample}$ = Volume of cell aliquots, which was used for the sample preparation.

3.4.3.3 Initial formation of catecholamines

The natural formations of catecholamines were measured in the culture of *Tetrahymena* cells in 96-well plates. 50 µL exponential growing cell containing 5×10^4 cells/mL were added to 200 µL PPY-medium into the well plates. The plates were incubated for several exposure times (6 h, 10 h, 21 h, 24 h, 32 h, and 48 h) at 28^{o}C in an incubator. Initial cell concentration in the system was adjusted to 1×10^4 cell/ml according to the OECD guidelines. After each incubation time, 200 µL cell culture aliquot was transferred into SPE-cartridge and extracted catecholamines.

3.4.3.3.1 SPE-Extraction of catecholamines

200 µl experimental cell aliquots from 96-well plates after required incubation time were harvested and then transferred to solid phase extraction (SPE-cartridge). Clean-up of catecholamines samples is carried out according to the manufacturer guide line (ClinRep® HPLC Complete kit, Recipe, Germany). This technique is widely described in the literature (Koller, 1988). However, the complete extraction of catecholamine was designed by extraction, washing and elution.

Extraction

200 µl *Tetrahymena* culture, 750 µL 2M Tris-HCL and 50 µL $Na_2S_2O_7$ (10 g/L) were mounted in 2 mL eppendorf tube. Then it was subjected to ultrasonication of five cycles (Bandelin Sonoplus GM 70, Germany) for the lysis of cell. $Na_2S_2O_7$ was also added to prevent the destruction of catecholamines by oxidation during extraction. The liquids of eppendorf tube and 50 µl internal standard (=500 pg) were transferred into SPE-cartridges by opening upper cap. The cartridge contains 22 mg activated Al_2O_3 in 0.5 mL 2M Tris-HCL.

Then the SPE-cartridges was closed by cap and vortexed for 10 minutes to bind catecholamines onto the surface of Al_2O_3. Afterwards the upper cap was opened and the lower cap of the sample preparation column was removed. The liquid phase of the column was sucked away using a vacuum manifold (Supelco visiprep^{TM}DL, USA).

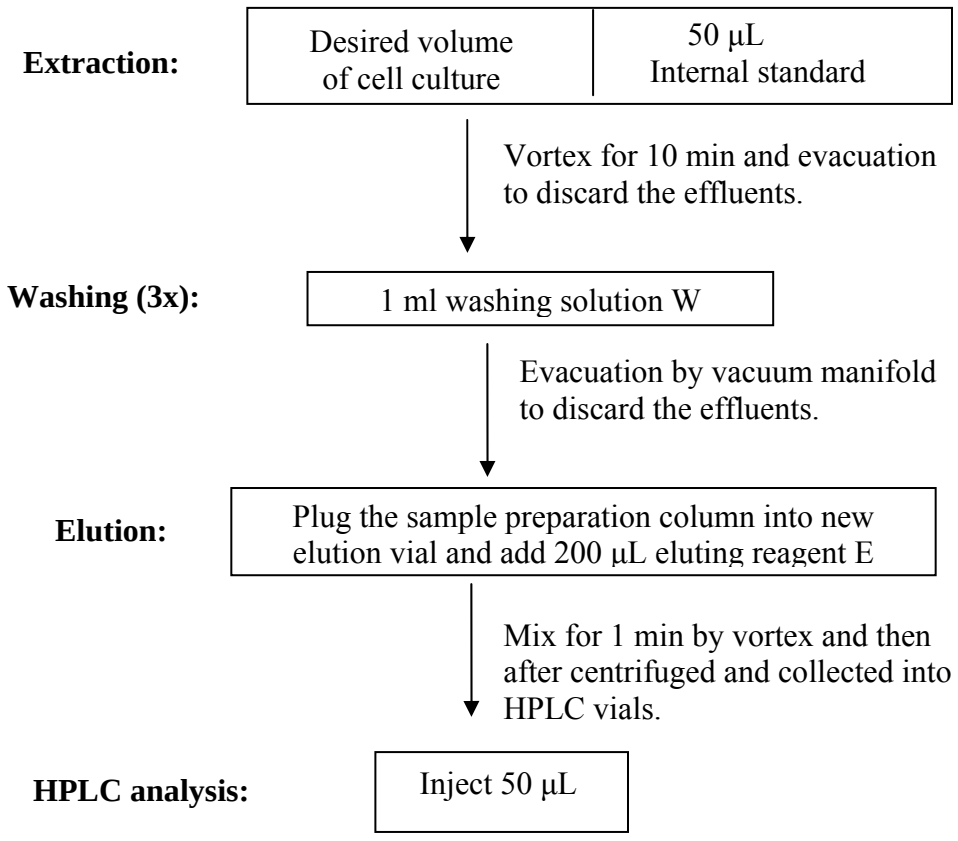
Washing

The sample preparation column was washed three times with washing solution W (1 mL each time). Afterwards, it was again evacuated using a vacuum manifold and dried by centrifugation (Hettich universal, Austria) for 1 minute at 1000×g to finally discard all liquid content from column.

Elution

200 µL of the eluting reagent E was added into dry cartridges which consequently vortexed for 2 minutes. Furthermore, SPE-cartridge was centrifuged for 1 minute at 1000×g. Finally, eluents of the cartridge were collected into HPLC vials. The eluted samples were stored at -80^{o}C until analysis.

Flow diagram



3.4.3.4 Degradation of catecholamines

The catecholamines known as dopamine, adrenaline and noradrenalin were exposed to both in the PPY medium and cell culture separately. At the beginning of the test the stock solutions of experimental compounds were diluted with PPY medium to a final concentration of 0.2 ppm. However, the 96-well plates were prepared consisting of 150 µL diluted solution and 100 µL PPY medium or 100 µL exponential growing *Tetrahymena* culture (2-3×10^4 cell/mL) separately to have 250 µL reaction volume; thus ultimately reached the final concentration of 0.12 ppm. This test concentration is equivalent to 783 nmol, 655 nmol and 709 nmol for dopamine, adrenalin and noradrenalin respectively. Afterwards the plates were gently shaken and incubated at 28^{o}C for 48h. At the end of incubation 200 µL aliquots were spiked into solid phase extraction cartridges. 200 µL effluents were collected into HPLC vials which moved to consequent measurement of catecholamines with HPLC-ED. The extraction procedure was executed similar as for section 3.4.3.3.1.

3.4.3.5 Investigation of catecholamine metabolites

Metabolites or products generated from *Tetrahymena* cells were observed in the 96-well plate setup in association with exogenous chemical pressure of all representative catecholamines. This experiment was carried out in comparison to higher number of exponential growing cells. Enhanced cell number would be expected to show a result of relatively higher concentration of metabolites. Two different concentrations were selected for this purpose. One is at very low physiological concentration of 0.12 ppm and another is at higher concentration of 8.0 ppm. However, these two optima concentrations chosen were adjusted after checking exposure and experience of many other concentrations. Stock solutions of catecholamines were diluted with PPY medium to create a working concentration of 0.3 ppm and 20 ppm respectively. Well plates were prepared and consisting of 150 µL log-phase culture (5×10^4 cell/ml) and 100 µL diluted compounds which ultimately reached the expected two different concentrations of catecholamines. Now the designed well plates were incubated for 48 h at 28^{o}C. After incubation, 10 µL aliquot of chemically exposed cells were mounted into the SPE-cartridge for clean-up. Here, a small scale aliquot was chosen to maintain a low concentration of catecholamine in the eluent of the SPE-cartridge. Higher concentration potentially caused the memory effects at the HPLC-ED. However, due to the exposure of every member of catecholamines (L-DOPA, dopamine, noradrenaline, adrenaline and

DOPAC), only dopamine and L-DOPA were responsible for the production of a metabolite at the higher concentration of 8 ppm.

The same experiment with dopamine was conducted in combination with 1 ppm ascorbic acid as an anti-oxidant. Furthermore, exponential growing culture was separated into two fractions; 100% cell free medium and cell containg medium. Cell separation is carried out by the centrifugation of culture in 1.5 ml eppendorf tube at 8,000 rpm for 10 min. The supernatant of cell was again filtered through a mini elute column for DNA (Qiagen, Germany) to obtain cell free medium. On the other hand, cell pellets were filtered using a membrane filter (pore size 3.0 µm) followed by two washing steps with 2ml phosphate buffer saline (PBS). Now, the washed cells were collected again in the fresh PPY medium. The cell free medium was also boiled to denature all proteins which could be liberated from *Tetrahymena* cells due to its rapid growth. In this case, it was expected that some proteins may be released from growing cells which may interfere the metabolite formation. However, due to the boiling of cell free medium all proteins are destroyed and should not affect metabolite generation. These three subsets of samples (cell free medium, cell in fresh medium and boiled cell free medium) were used for testing on the presence of the dopamine metabolite. 100 µl dopamine of 20 ppm concentration was exposed to 150 µl of these aliquots and evaluated accordingly as above as mentioned.

3.4.3.6 Determination of molecular weight and formula of new metabolite employing LC-MS and FTICR-MS.

3.4.3.6.1 Preparation of sample

The acclimatized pre-culture of *Tetrahymena* cells (1-1.5×10^5 cell/mL) was used for generating dopamine metabolite with exogenous exposure of dopamine. The stock solution of dopamine was diluted in PPY-medium to 20 ppm. Well-plates were prepared according to the section 3.4.3.5. Afterwards, the plates were incubated at 28^{o}C for 48 hours. Then, 100 µl cell suspensions were transferred into eppendorf tube and diluted to 1 mL by 2M Tris HCl buffer. These diluted cells are subjected to five cycle ultrasonication. Now, 100 µL aliquot were transferred into SPE cartridge. The solid phase extraction of metabolite was described in the section 3.4.3.3.1. Finally, 200 µl eluent of SPE-cartridge was collected into a glass vial for the determination of molecular mass analysis by UPLC-MS. Samples were preserved in refrigerator at -80^{o}C until measurement and analysis.

3.4.3.6.2 Condition of Liquid Chromatography/Mass Spectroscopy

A NanoAcquity UPLC system (Waters, Milford, USA) was employed for sample separation on a nano-scale column Atlantis dC18 UPLC, particle size 3µm, 75µm × 150mm, and column temperature 40°C. Auto sampler temperature was maintained to 10°C. The automatic injection volume was 1µL. The sample was pre-concentrated on a trap column (Symmetry C18 UPLC, particle size 5 µm × 180 µm× 20 mm, Waters, acetonitrile/water 5:95 or 2:98 v/v, flow 4 µL/min for 2-3 min) prior to online transfer to the analytical column. Gradient conditions for chromatography were: initially 0-3 min acetonitrile/water 5:95 or 2:98 v/v, 0.2 µL/min; to 10:90 v/v, after 5 min flow increased to 0.3 µL/min; to 100% acetonitrile after 12min and hold for 8 min finally back to initial conditions and equilibration for 15 min. Acetonitrile was LC/MS quality, water was highly purified by sub-boiling in a quartz apparatus; all solvents and compositions containing 0.2% of formic acid. The column outlet is directly connected to the emitter capillary of the nano-electrospray ionization source of the mass spectrometer

The outlet capillary of the chromatography column was connected to the nanospray source of a Q-Tof 2 mass spectrometer (Waters-Micromass, Manchester, UK) applying positive ionisation and PicoTip® emitter (New Objective, Woburn, USA). Fundamental instrument parameters were: capillary voltage 2kV, cone 16V, source temperature 100°C, TOF 9.1kV, MCP 2.3kV with resolution of 7500. Scan function parameters were: mass range 100 – 300amu, scan time 2sec, inter-scan time 0.1sec, data format continuum. MassLynx 4.1 software (Waters-Micromass) was used for control and data treatment in the NanoAcquity™ UPLC™ and Q-Tof2 systems.

3.4.3.6.3 Freeze drying of the cell extract

The eluent of SPE-cartridge was concentrated by freeze dryer due to the presence of very low concentrations of the metabolite. Low concentrated metabolite is difficult to analyze by FTICR/MS. Therefore, a concentrated (ppm level) metabolite is a pre-requisite for FT/MS analysis. That is why; the eluents of cell extract was reduced by freeze drying without distortion of metabolite. 200 µl cell extract can be collected from each cartridge. So, the extracts of 10 cartridges were gathered in an eppendorf tube which made the volume 2 mL. Afterwards, this 2 mL cell extract was condensed and reduced to 200 µL employing freeze

dryer (Freezemobile 5, Vitros, USA). The freeze dryer was adjusted to condensation temperature at -55^{o}C, vacuum 20 millitorr and volt 215V. This condensed cell extract with higher concentration of metabolite was infused into the FT-MS with higher concentration of metabolite.

3.4.3.6.4 Fourier Transform Ion Cyclotron Resonance-Mass Spectroscopy

Ultra high resolution mass spectrometer for the assignment of molecular formula was acquired from Bruker (Bremen, Germany) APEX Qe ICR-FT/MS with 12 T superconducting magnet and an Apollo II electrospray (ESI) source, whereas high-resolution spectra are acquired with an Apollo II ESI source. The sample was injected with methanol into micro electrospray source at a flow rate of 120 ml/h with a nebulizer gas pressure of 20 psi and a drying gas pressure of 15 psi (250^{o}C). Metabolite was analyzed by positive ionization. Spectra were externally calibrated on clusters of arginine (10 mg/L in methanol), and calibration errors in the relevant mass ranges are always below 200 ppb, The spectra were acquired with a time domain of 4 mega word (where 1 data word corresponds to 32 bits) and zero filled to a processing size of 2 mega words. A sine apodization was performed before Fourier transformation of the time domain transient.

3.5 Identification of dopamine receptor in *Tetrahymena thermophila* by fluorescent ligands.

3.5.1 Chemicals:

Two fluorescent ligands were used for this experiment. Dopamine D1 selective receptor agonist SKF-38393, R(+)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol and antagonist SCH 23390, 7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine were purchased from Sigma-Aldrich Chemie GmbH Laboratory, Germany. Both ligands were derivatized by fluorescent rhodamine-dye (Monsma et al., 1989). Selective D_2 dopamine receptor antagonist, 2-Methyl-spiperone hydrochloride and dopamine antagonist, (+)-butaclamol hydrochloride were also purchased from Sigma-Aldrich Chemie GmbH Laboratory, Germany. The stock solutions of these compounds were prepared in Millipore water according to the solubility instruction of manufacturer's guideline.

3.5.2 Fluorescent microscopy

The photograph of cell was captured by two different fluorescent microscopes. One is Axioplan 2 connected to Camera, AxioCam MRm (Carl Zeiss, Germany) with 546/590 nm an excitation/emission filter for rhodamine fluorophore. The other one is the laser scanning microscope, LSM 510 META (Carl Zeiss, Germany). Argon ion laser, 488 nm excitation and band pass (BP) filter 500-550 nm emission were used. LSM software version 3.2.SP2 was used for the image analysis. Image was magnified to 40 times by C-Apochromat Water Immersion Lens (Helium Neon Laser 543 nm excitation and LP 560 emission).

3.5.3 Protozoan cell and culture medium

The ciliated unicellular protozoan *Tetrahymena thermophila* SB210 cells were anexically grown in 40 mL sterile PPY medium in Erlenmeyer flasks. Flasks were covered by silicone rubber (fine porous) heads. Cells were acclimatized in PPY-medium with two successive re-seeding of 400 µL cell aliquot in 40 mL fresh medium. The cell density increased exponentially up to 1-4×10^5 cells/mL after overnight incubation.

3.5.4 Exposure of dopamine ligands to the *Tetrahymena thermophila*

The experiment was conducted in 96 well plates. Each well contained 250 µl test volume. Initial cell concentration of this experiment was controlled to 1×10^4cells/mL being incubated at 28^{o}C. Five mounted concentrations; 5 µM, 10 µM, 20 µM, 40 µM and 80 µM for each ligand were selected for the experiments. The rhodamine-labeled dopamine receptor agonists SKF-38393 was exposed to the *Tetrahymena* cells and incubated for different period of times. The convenient binding time for the agonist was considered for 3 h. This optimal incubation time was chosen over several exposures. After the suitable incubation period, 200 µl cell-suspensions were transferred into 1.5 mL eppendorf tubes. The cells were fixed with 10% formaldehyde and washed twice steps with PBS. Afterwards, 6µl cell pellets were allocated on glass slides to investigate the fluorescent signal employing fluorescence microscopy. Just prior to examination, the slides were covered with cover slips and a drop of immersion oil was added on the top. Then, cells were observed using the fluorescence optics of a Zeiss photomicroscope-2 with 546/590 nm excitation/emission filters for the rhodamine fluorophore. Two-dimensional photomicrographs were captured by an auto camera with 40×magnification. The specific binding of the fluorescent probe to dopamine receptors was

assessed with competition between D1 specific agonist SKF-38393 and antagonist SCH 23390. Both ligands were labelled with rhodamine. They were exposed for the same time in the cells at the similar five nominal concentrations and incubated for 3 hours. In addition, the same experiment was conducted with SKF-38393 and common non-labelled dopamine receptor antagonist (+)-butaclamol. Butaclamol is an active enantiomer which inhibits agonist binding to dopamine receptor. Furthermore, the binding of SKF-38393 to the D1 type receptor was faithfully cross checked using D2 antagonist, spiperone. These two ligands were exposed to the cells at the same molar concentrations and incubated such as mentioned above. Later, the cross-reactivity of SKF-38393 to D2 receptor was scrutinized under the same experimental setup. Additionally, one more experiment was considered with cells previously stimulated by L-DOPA. Prior to 24 h experiments 2 ppm L-DOPA was added in the cells. L-DOPA causes the stimulation of dopamine receptors in higher animals. This conveniently treated cell was used for the exposure of SKF-38393.

The 40 µM concentration of SKF-38393 exhibited the higher binding sensitivity to D1 receptor after 3 h incubation. This binding of SKF-38393 was used for further assessment employing laser scanning microscope (LSM). The LSM capture photograph from a different plane; thus indicates the binding location of agonist in the cell. Slide preparation carried out as above mentioned.

3.6 Toxicity and geno-biotic transfer of 5-FU between aquatic microorganisms

3.6.1 Chemicals

Radioactive [2-^{14}C] 5-fluorouracil (50µCi/500µl and concentration 245.5 µg/mL or 1.88 mM) was purchased from Hartmann Analytic GmbH, 38124 Braunschweig, Germany. The activity of the radioactive 5-fluorouracil is 532 mCi/mM. Permafluor-E, CARBO-SORB, and Utima Gold XR were purchased from PerkinElmer LAS GmbH**,** 63110 Rodgau-Jügesheim, Germany. 5-fluorouracil and all the compounds required for the preparation of algae culture medium were purchased from Sigma-Aldrich Chemie GmbH Laboratory, Germany. The stock solution of 5-fluorouracil was prepared in the Millipore water which gives a clear solution up to 10 mg/mL.

3.6.2 Cell growth inhibition test of 5-FU to microalgae, *Scenedesmus*

A new batch culture was established by transferring algae from a culture in the late exponential growth phase into a sterile 250 mL Erlenmeyer flask which consisted of 100 mL sterile medium. Initial cell density was adjusted to 1×10^4cells/mL (OECD, 201). Six nominal concentrations (200, 100, 50, 25, 12.5 and 6.25 ppm) of 5-FU were used for this study. Samples are loaded under sterile condition. Test flask were closed by a scuro cap containing cuvate (100-QS, Hellma optic GmbH jena, Muhlenstr. 20, germany) of light path 10 mm. this cuvate advantages the direct measurement of cell density with a spectrophotometer without opening the flask, thus maintain the sterile condition. At the time of cell concentration measurement, the flasks are turn in to fill the cuvate by test liquid and consequently penetrated into the measurement chamber of spectrophotometer. Cell growth was verified by the measurement of optical density at 660 nm (Spectrophotometer Unicam 5675, England). The test flasks were placed on a culturing system that maintains continuous shaking and uniform illumination at 21-25^{o}C temperature. In addition the cells were also counted with a Neubauer counting chamber (Brand Wertheim, Germany). Optical densities of the cell are increased proportionally with cell growth and increase of biomass. Therefore this correlation is used for the determination of toxicity of 5-FU. Here, the exposures of the cells were monitored after 24 h and 48 h respectively. The percentage of inhibition of cell growth rate for each treatment replicate (n = 3) was determined in comparison with untreated control as described in section 3.4.2 according to the equation number 1 and 2. A concentration-response relation curve was constructed by plotting the percentage of inhibition against the logarithm of applied concentration of the substance. The effective concentration (EC_{50}) of 5-FU was read from the eye fitted curve.

3.6.3 Cell growth inhibition test of 5-FU in protozoan, *Tetrahymena*

The stock solution of 5-FU was directly diluted into PPY-medium. Prior to the inoculation of cell, the *Tetrahymena* cell density was adjusted to 5×10^4 cells/mL. Cell inoculation was conducted inside a sterile clean bench (Heraeus LaminAir® HBB 2448 S, North Germany). 200 µL 5-FU samples and 50 µL exponential growing *Tetrahymena* cell were taken in 96-well plates. The applied concentrations of 5-FU were governed from 125 ppm down to 0.24 ppm as 50% dilution series (e.g. 125, 62.5, 31.75 etc). Well plates were shaken gently to homogenize distribution of cells through the test environment and placed in an incubator at 28^{o}C. Optical density (OD) of cell was measured for zero h, 24 h and 48 h respectively with spectrophotometer (SLT-spectra, Salzburg, Austria) at 550 nm. OD of the cell is

proportionally increased with the growth of *Tetrahymena* cells. The percentage of cell growth inhibition for each treatment concentration was calculated as same for evaluation of algae toxicity according to the equation number 1 and 2. Finally, the effective concentration EC_{50} was obtained from concentration-response relationship curve.

3.6.4 Bioaccumulation test of 5-FU in algae

Radio-labelled [2-^{14}C] 5-FU consisted of 50,000 disintegration per min (DPM) corresponding to 22.53 nCi (55.29 ng) was considered for testing bioaccumulation of 5-FU. This amount of radioactive 5-FU was transferred into 200 mL algae culture (exponentially growing cells) in 500 mL Erlenmeyer flasks. Initial cell concentration was adjusted to 1×10^4 cells/mL and incubated for 24 h as for section 3.5.2. The applied 5-FU concentration was supposed to have no toxic effect or may have very little toxicity but does not affect cell growth. After 24 h, 20 mL algae cell suspension was centrifuged at 4500 rpm/min for 30 minutes followed by twice sequential washings with PBS. Afterwards, cell pellets were filtered on a membrane filter (Sartorius Typ SM II 302, diameter 47 mm, pore size 3.0 μm) and washed twice step with PBS. Now the algal cells including filter paper (dried) were ignited in pure oxygen applying a sample oxidizer (model 307; Packard, Meriden, CT, USA). The oxidised samples were collected in a white plastic scintillation vial within 15 mL of Permafluor E and counted radioactivity employing liquid scintillation counter (Liquid Scintillation Analyzer, Tri-carb 1500 TR, Packerd, Meriden, CT, USA). Each count consisted of mean value of three individual counts for 10 min. Furthermore, 5 mL supernatant of cell culture in 10 mL Ultima Gold XR was considered to count radioactivity for free labelled 5-FU in the medium. The bioaccumulation factor of 5-FU is the ratio of the weight of accumulated substance in the algae (per Kg) to the un-accumulated substance in the test medium (per L). It was calculated according to the following equation (5). The same experiment was conducted for untreated control cells.

$$\text{Bioaccumulation factor (BAF)} = \frac{\text{Amount of 5-FU accumulated in the algae}}{\text{Amount of un-accumulated 5-FU in medium}} \quad \ldots\ldots\ldots\ldots 5$$

3.6.5 Distribution of labelled 5-FU to DNA and total RNA in microalgae

Radio-labelled [2-^{14}C] 5-FU consists of 222000 DPM equivalents to 100.01 nCi (245.5 ng) was added to 100 mL algal culture in 250 mL Erlenmeyer flask. Initial cell concentration was adjusted to 22×10^4 cells/mL and incubated for 24 h as similar condition of section 3.5.2. Flasks are closed by scuro cap until the incubation thus maintains sterile condition. 30 mL cell culture is centrifuged in a glass tube at 4500 rpm/min for 30 minutes. The cell pellets were washed twice with PBS following centrifugation and finally collected in a 1.5 mL eppendorf tube. DNeasy Plant Mini Kit and RNeasy Plant Mini Kit (Qiagen, Germany) were used to extract DNA and total RNA respectively according to manufacturer's guidelines. In both cases of extraction, elution volume was chosen for 200 µL to achieve maximum quantity of genetic material. Now, 50 µL DNA/RNA extracts were mounted on a cellulose cap and incinerated them in an oxidizer. The gaseous oxidation products were collected in 15 mL Permafluor E in a vial and counted radioactivity. Also, the extraction waste of DNA (2.2 mL), total RNA (2.3 mL) and the mini spin column were collected and considered for count radioactivity. In addition 100 µl extraction wastes are ignited on cellulose paper and oxidized samples collected in 15 mL Permafluor E. Furthermore, 100 µl cell supernatant was transferred into 15 mL Ultima Gold XR for counted radioactivity of free 5-FU in the medium.

3.6.5.1 Purification of microalgae total DNA

DNeasy Plant mini kit provides the pure total DNA (genomic, mitochondrial and chloroplast) from microalgae. The procedure of DNA extraction is described as follows:

1. Added 400 µl Buffer AP1 and 4 µl RNase of stock solution (100 mg/ml) to the washed microalgae cell in the 1.5 mL eppendorf tube (≥ 100 mg) and vortex vigorously.
2. Incubated the mixture for 10 min at 65°C. Mix 2 or 3 times during incubation by inverting tube. This step lyses the cells.
3. Added 130 µl Buffer AP2 to the lysate, mixed, and incubated for 5 min on ice.
4. The lysate was centrifuged for 5 min at 20,000 × g (14,000 rpm).
5. Pipeted the lysate into the QIAshredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at 20,000 × g (14,000 rpm).
6. Now transferred the entire flow-through fraction from step 5 into a new tube without disturbing the cell-debris pellet.
7. Added 1.5 volumes of Buffer AP3/E to the cleared lysate, and mixed by pipetting.
8. Pipeted 650 µl of the mixture from step 7 including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 ml collection tube. The

whole column was centrifuged for 1 min at 6000 × g (8000 rpm) and discarded the flow-through.

9. The step 8 was repeated with remaining sample. Flow-through and collection tube were discarded.
10. DNeasy Mini spin column was placed into a new 2 ml collection tube and added 500 µl Buffer AW and centrifuged for 1 min at 6000 × g. Flow-through was discarded and reused the collection tube in step 11.
11. Added 500 µl Buffer AW to the DNeasy Mini spin column, and centrifuged for 2 min at 20,000×g to dry the membrane.
12. Finally, the DNeasy Mini spin column was transferred to a 1.5 ml and pipeted 200 µl Buffer AE directly onto the DNeasy membrane. Incubated for 5 min at room temperature (15–25°C), and then centrifuged for 1 min at 6000 × g to elute the pure DNA. This purified total DNA was stored in the refrigerator at -80^{o}C until measurement.

3.6.5.2 Purification of microalgae total RNA

The RNeasy plant mini kit allows the purification of total RNA (mRNA, rRNA and tRNA) from microalgae. The procedure is described as follows:

1. 450 µl Buffer RLT or Buffer RLC was added to 1.5 mL eppendorf tube containing microalgae cell (≤ 100 mg) and vortex vigorously.
2. The cell lysate was transferred to a QIAshredder spin column (lilac) placed in a 2 mL collection tube, and centrifuge for 2 min at full speed. Supernatant of the flow-through was carefully transferred into a new 2 mL eppendorf tube without disturbing the cell-debris pellet in the collection tube. Only this supernatant was used in the subsequent steps.
3. Added 0.5 volume of ethanol (96–100%) to the cleared lysate and mixed immediately by pipetting, and then proceeded immediately to step 4.
4. The sample (usually 650 µl) including any precipitate that may have formed was transferred into an RNeasy spin column (pink) placed in a 2 ml collection tube. The lid was closed gently and centrifuged for 15 s at 8000 × g (10,000 rpm). The flow-through discarded.

5. Added 700 µl Buffer RW1 to the RNeasy spin column. Closed the lid gently, and centrifuged for 15 s at 8000 × g to wash the spin column membrane. The flow-through was discarded.
6. 500 µl Buffer RPE was added to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 8000 × g to wash the spin column membrane and discarded the flow through. This step was repeated one more.
7. Placed the RNeasy spin column in a new 2 ml collection tube, and discarded the old collection tube with the flow-through. After close the lid gently, the column was centrifuged at full speed for 1 min.
8. The RNeasy spin column was place in a new 1.5 ml collection tube and added 200 µl RNase free water directly to the spin column membrane. After close the lid gently, it was centrifuged for 1 min at 8000 × g to elute the RNA. This purified total RNA was stored in the refrigerator at -80^{o}C until measurement.

3.6.6 Genetic transfer of labelled 5-FU into Protozoan

In this experiment, both radioactive algal DNA and total RNA together are fed to *Tetrahymena pyriformis* cells as enriched genetic materials in 40 mL PPY-medium within a 300 mL Erlenmeyer flask with porous stopper. Initial cell concentration was adjusted to 1×10^{4}cells/mL (cell growth acclimatized as previously) and incubated for 24 h and 48 h respectively at 28^{o}C. Afterwards, 5 mL cell culture was centrifuged in a glass tube at 5500 rpm for 10 min followed by twice washing steps with PBS. Cell pellets was then filtered through a membrane filter and washed again twice with 5 mL PBS. Then the *Tetrahymena* cells in addition with membrane filter was considered to combustion in oxidizer. Now, the oxidized sample was collected 15 mL Ultima Gold XR and counted the radioactivity as described above. A control experiment was also conducted with PPY medium without inoculating *Tetrahymena* cells.

3.6.7 Reintegration of labelled 5-FU into protozoan DNA

Radioactive genetic materials of algae was introduced into the *Tetrahymena pyriformis* culture and incubated for 48 h as described already. *Tetrahymena* cell was harvested as before and collected into 1.5 mL Eppendorf tubes. Afterwards DNA of *Tetrahymena* was extracted using DNeasy tissue kit (Qiagen, Germany) according to manufacturer's guidelines. 50 µl

DNA extract was incinerated in an oxidizer and sample collected in 15 mL Ultima Gold XR followed by counting of radioactivity as already described.

3.6.7.1 Purification of total DNA of *Tetrahymena*

DNeasy Tissue Kits are designed for rapid purification of total DNA (e.g. genomic, mitochondrial, and pathogen) from a variety of sample sources. This kit was used for the purification of total DNA for *Tetrahymena*:

1. 5 mL *Tetrahymena* cell culture was washed as similar for the section 3.5.6 and transferred the cel pellet into 1.5 mL eppendorf tube. Now, added 180 µl Buffer ATL.
2. Added 20 µl proteinase K and mixed thoroughly by vortexing, and incubated at 56°C until the tissue is completely lysed.
3. Again vortex for 15s, added 200 µl Buffer AL to the sample, and mixed thoroughly by vortexing. Then, 200 µl ethanol (96–100%) added and mixed thoroughly by vortexing.
4. The mixture from step 3 (including any precipitate) was pipeted into the DNeasy Mini spin column placed in a 2 ml collection tube and consequently centrifuged at 6000 × g for 1 min. Discarded the flow-through and collection tube.
5. DNeasy Mini spin column was placed in a new 2 ml collection tube, and added 500 µl Buffer AW1, and centrifuged for 1 min at 6000 × g. Discarded the flow-through and collection tube.
6. Again, DNeasy Mini spin column was placed in a new 2 ml collection tube and added 500 µl Buffer AW2, and centrifuged for 3 min at 20,000 × g to dry the DNeasy membrane. The flow-through and collection tube were discarded.
7. Now, the DNeasy Mini spin column was placed in a clean 1.5 mL eppendorf tube, and pipeted 200 µl Buffer AE directly onto the DNeasy membrane. It was incubated at room temperature for 1 min, and then centrifuged for 1 min at 6000 × g to elute the purified total DNA.

3.7 The effect of perfluorinated surfactants to *Tetrahymena*

3.7.1 Chemicals

The perfluorinated compounds e.g. perfluorooctanoic acid (PFOA) from Chempur, Karlsruhe, Germany, and perfluorooctane sulfonate (PFOS) in form of tetraethylammoium salt from Sigma-Aldrich, Taufkirchen, Germany were purchased. In addition the perfluorinated telomere alcohols such as 4:2 FTOH, 6:2 FTOH, 8:2 FTOH and 10:2 FTOH were purchased

from Sigma-Aldrich, Germany. All the compounds were dissolved in methanol-water (1:4, v/v) using pure methanol (Sigma-Aldrich, Germany) and Milli-Q water. The concentrations of stock solution of PFCs are prepared to 1000 mg/L. On the other hand, the initial concentration of stock solutions of 4:2 FTOH, 4:6 FTOH, 8:2 FTOH and 10:2 FTOH were prepared to 10000mg/L, 8000 mg/L, 8000 mg/L, and 8000 mg/L respectively. However, the stock solutions were diluted by direct addition of cultivation medium prior to the test to achieve working concentration. Final methanol concentration in the samples never exceeded 2% and its toxic effect was subsequently subtracted from the data. Cytotoxicity Detection Kit^{PLUS} for determination of cell membrane damage was purchased from Roche Diagnostics GmBH, Mannheim, Germany.

3.7.2 Growth inhibition test

3.7.2.1 Cell growth inhibition of *T. pyriformis* to PFOA and PFOS

The acclimatized ciliates protozoan cell, *Tetrahymena pyriformis*, strain GL was used for this study. They were anexically grown in the PPY medium. However, the toxicity assay was performed as a critical dilution assay in 96-well plates by monitoring the increment of *Tetrahymena* cell as compared to an untreated control and methanol treated groups. One day old pre-experimental logarithmically growing culture (1-2×10^5 cells/ml) was added to the samples with PFOA and PFOS concentrations ranging from 0 to 100 mg/L and incubated at 28°C. Therefore, ultimate volume in the well of the plate was hold to 250 µl wherein cell concentrations were adjusted to 1×10^4 cells/ml. In addition, 2% methanol in PPY medium was also tested in *Tetrahymena.* The density of the cell population was measured spectrophotometrically at 550 nm using SLT Spectra microplate reader. However, the measurements were done after 24 and 48 hours of exposure. On the basis of optical densities of the samples a percentage of growth inhibition were calculated (the difference of OD of control and treated sample related to OD of control) according to the equations number 1 and 2. A concentration-response relation curve was drawn by plotting the percentage of inhibition against concentration of substance. The value of effective concentration (EC_{50} for PFOS and EC_{10} for PFOA, respectively) was evaluated from the eye fitted curve.

3.7.2.2 Cell growth inhibition of *T. thermophila* to perfluorinated alcohol

The fluorotelomer alcohols (FTOH) were diluted in PPY-medium under sterile condition, leading to final concentrations separately ranging from 0 to 500 ppm (for 4:2 FTOH), 0 to 250 ppm (for 6:2 FTOH), 0 to 40 ppm (for 8:2 FTOH) and 0 to 3.2 ppm (for 10:2 FTOH).

Toxicity assay was performed with critical dilution assay in 96-well plates by monitoring the increment of *Tetrahymena thermophila* cell as compared to the methanol treated group and untreated control group. One day old pre-experimental logarithmically growing cell (approx. $1\text{-}2\times10^5$ cells per mL) was considered as suitable cell. 200 µL of 4:2 FTOH, 6:2 FTOH, 8:2 FTOH and 10:2 FTOH and 50 µL cell aliquots were taken into the well-plates. These designed well plates were incubated at 30^{o}C in the incubator. The density of the cell population was measured spectrophotometrically at 492 nm using SLT spectra microplate reader. However, in this case the filter of the spectrophotometer was changed to 492 nm due an error of the instrument but this filter can also faithfully measure the differences of cell growth between test and control sample. The cell growth inhibition was calculated as same to the section 3.4.2 according to the formula 1 and 2.

3.7.3 LDH leakage assay of *T. pyriformis* to PFOA and PFOS

LDH leakage from cells to the culture medium was determined using a 'Cytotoxicity Detection Kit^{PLUS}' by colorimetric assay. Control experiments showed that solvent exposure including 2% methanol did not affect total LDH activity, therefore LDH leakage was expressed as a percentage of LDH release from cells lysed with PFCs. The *Tetrahymena pyriformis* cells were pre-cultured and acclimatized in the culture medium to achieve exponentially growing cell. These suitable cells were diluted to an optimum cell concentration of 1×10^4cell/ml. There are eight different nominal concentrations (100, 90, 80, 70, 60, 50, 40 and 30 ppm) of both PFOA and PFOS were considered for this assay in the 96 well plates. The compounds tested, at sufficiently high concentrations, had the capacity to interfere with cell membrane causing as a consequence leakage of the membrane. However, the experiment was followed according to manufacturer guidelines of this kit (Roche Diagnostics). The cytotoxicity of the surfactants was calculated with respect to the following formula.

$$\text{Cytotoxicity (\%)} = \frac{\text{Exp. Value} - \text{low control}}{\text{High control} - \text{low control}} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad 6$$

3.7.3.1 Cytotoxicity assay procedure

The exponential growing cell was washed twice with assay medium and adjusted the cell concentration to 1×10^4 cell/mL. Now, *Tetrahymena* cell was exposed with PFOA and PFOS in the 96 well plates and incubated for 24 hours. The LDH leakage of the cell was determined as follows:

1. 50 µL cells were transferred into 96 well plates. Afterwards 50 µl test substance (n=3) was added in the cell of well plate, and incubated in an incubator (37^oC, 5% CO_2, 90% humidity) for 24 hours.
2. On the same plate; the background control, low control, high control, substance control I and substance control II were prepared in triplicate according to the guideline.
3. To each of the well that contains high control sample added 5 µL lysis solutions.
4. Incubate the plate for an additional 15 min.
5. 100 µl reaction mixtures was added to each well on the 96-well plate and incubated for upto 30 min at room temperature in dark place.
6. The reactions are stop with the addition of 50 µl stop solution to each well on the 96 well plates. Shaken the plate for 10 s.
7. Now, the absorbance of the sample was measured at 492 nm using spectrophotometer (SLT sectra, Salzburg, Austria).
8. Calculated the cytotoxicity for the sample according to formula no. 6.

3.7.4 The effect of FTOH on macronucleus of *Tetrahymena thermophila*

FTOHs were exposed to the *Tetrahymena thermophila* and incubated for 48 hours. In addition the cells were also incubated to the negative control (methanol) and positive control (phenol). Afterwards, 200 µL cells were stained with 5 µL acridine organe (AO) dye (Sigma-Aldrich, Taufkirchen, Germany) and waited for 20 minutes. The stained cells were washed twice or three times with PBS. Furthermore the stained cells were fixed with 10% formaldehyde (from Sigma) and examined the nucleus employing fluorescent microscope (Axioplan 2). Acridine orange binds to DNA of macronucleus which appeared green at 488 nm due the fluorescence of the dye.

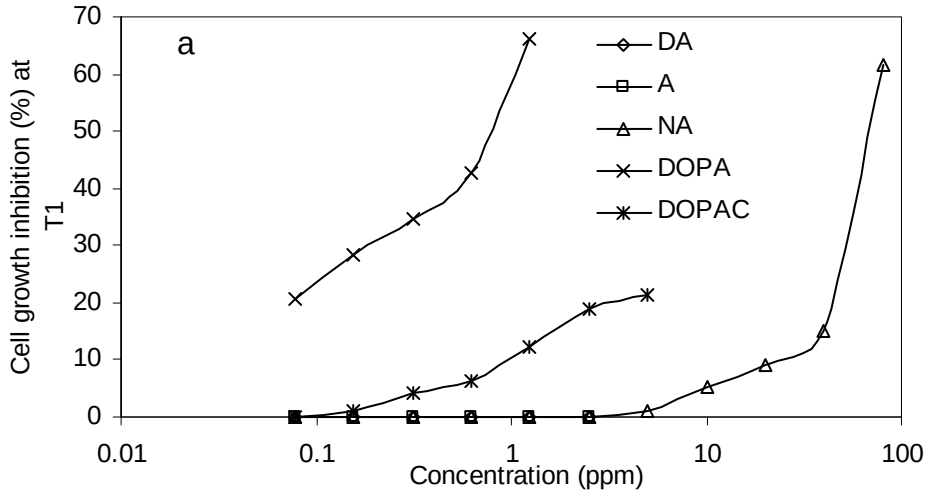
3.8 Feeding effect of nano TiO_2 particles to *Tetrahymena thermophila.*

Nanoparticles (NPs), titanium dioxide (TiO_2, size<10 nm) was purchased from Sigma-Aldrich, Taufkirchen, Germany. Its stock solution was prepared directly in the sterile PPY-medium. But the problem is faced that the nanoparticles are insoluble in water and always have a tendency to sedimentation. The ultrasonication of the insoluble nanoparticles gives an unstable suspension. Therefore, prior to the experiment the stock solution of TiO_2 was always ultra-sonicated (Bandelin, Sonorex, RK510S) for 5 min to obtain a homogenous suspension. This experiment was conducted in the small Erlenmeyer flasks containing 18 mL of the suspension of TiO_2 in PPY with different TiO_2 concentrations (0, 50, 100, 200, 400 ppm) and 2 mL one day old pre-experimental cell culture or 2 mL of PPY (cell-free samples). Initial cell concentration was adjusted to 1×10^4cell/mL. Then the cells exposed by nanoparticles were incubated in the horizontal shaker in the dark at 28°C with 80 RPM for 48 hours. Due to the high specific weight and quick sedimentation of nanoparticles, the flasks were continuously shaken to have a contact between cell and TiO_2 during exposure. Cell-free samples were also shaken to check the rate of natural aggregation of the particles. After the incubation the cells are fixed by 10% formaldehyde (1:1, v/v) and mounted on the glass slide for microscopic examination (Axioplan 1, Carl Zeiss, Germany).

4.1 Catecholamine toxicity and haemostasis in *Tetrahymena pyriformis*

4.1.1 Determination of catecholamine toxicity

Tetrahymena underlies reduced cell growth in presence of catecholamines. The measurements of toxicity of catecholamines were conducted using critical dilution assay in 96-well plates by monitoring the depletion of cells as compared to an untreated control after 24 h (T_1) and 48 h (T_2). Effective concentration (EC_{10} and EC_{20}) of catecholamine was calculated at two different exposure times. However, effective concentration is the statistical estimation of the concentration of a toxicant in the environment of medium necessary to produce a particular effect in a vary large population under described condition and the values were determined using a graphic method (Litchfield and Wilcoxon, 1949) as shown in the Fig 4-1. Higher concentrations of catecholamines were restricted to be applied in PPY medium due to its unspecific colour reaction with the proteose peptone in the PPY-medium. These colour reactions may result from the very oxidative chemical environment and interfere with the actual counting of cells. Dopamine and L-DOPA exerted higher potential of colour reaction. However, lower concentration of dopamine and L-DOPA up to 10 ppm did not affect the performance of the test. EC_{10} and EC_{20} could be determined successfully for both exposure time except adrenalin (A) and dopamine (DA) as presented in the Fig. 4-1 and Table 4-1. All the tested compounds exhibited moderate acute toxicity at 48 h. L-DOPA and DOPAC showed significant cell growth inhibition for both exposure times. However, the EC values for dopamine and adrenaline were not determinable for 24 h exposure due to the insignificant result of cell growth inhibition. Surprisingly, noradrenalin and adrenalin exhibited elevated EC values corresponding to lower toxicity than other compounds.



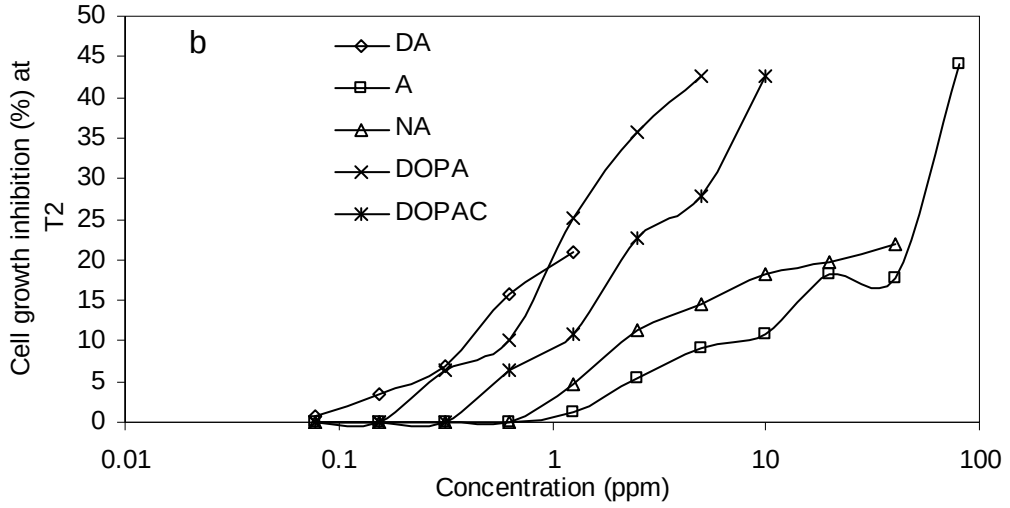


Figure 4-1: Dose-response curve of catecholamines in *Tetrahymena*. (a) and (b) illustrated % of cell growth inhibition versus log of concentration after 24h (T_1) and 48h (T_2) respectively. Data points represented from mean values of four replicates.

Table 4-1. Effective concentration (EC) of catecholamines in the *Tetrahymena pyriformis*.

Test compound	24 h (T1) inoculation				48 h (T2) inoculation			
	EC_{10}		EC_{20}		EC_{10}		EC_{20}	
	ppm	µmol	ppm	µmol	ppm	µmol	ppm	µmol
DA	n.d	n.d	n.d	n.d	0.39	2.55	1.1	7.18
L-DOPA	0.12	0.61	1.26	6.39	0.63	3.19	1.0	5.07
DOPAC	1.0	5.95	3.31	19.7	1.23	7.31	2.09	12.4
A	n.d	n.d	n.d	n.d	7.76	42.4	40.7	222
NA	20.4	121	41.7	246	2.19	12.9	24	142

Note: Not-determinable (n.d)

This effect on the cell growth indicated that DA and L-DODA were more active to inhibit the cell reproduction as compared to others. DOPAC showed moderate cell growth inhibition. Adrenalin and noradrenalin presented less interference to the growth of *Tetrahymena* cell. Dopamine adrenalin and noradrenalin are highly biodegradable (probably in terms of uptake through growth medium and not excreted as by-product). They could not exist through out the exposure time but may introduce some chemical signal such as ROS in the cell. Those chemical signals may cause the interference of cell division via inability of DNA enzymes to perform genetic materials reproducing for cell division. There are many investigations have been reported that catecholamines are considered as contributing factors that generate ROS due to its unstable catechol moiety and ultimately produce reactive quinone molecule. In that case DA exhibits a higher rate of oxidation as compared to adrenaline and noradrenaline indicating higher toxic potential of dopamine (Graham, 1978). Furthermore, DA and L-

DOPA, through auto- and enzymatic oxidation can generate a variety of cytotoxic oxygen radical species including superoxide, hydrogen peroxide (H_2O_2), semiquinones and quinones that are capable of modifying cellular macromolecules (Graham *et al.,* 1978). It was also claimed that DA-quinone and catecholamine-quinone itself is directly cytotoxic and genotoxic in addition to its significant role in generating ROS (Alan *et al.,* 1999). Therefore, the formation of free radicals can cause lipid peroxidation of cell membranes and induce dysfunction of the mitochondrial electron transport chain (Olano, 1993; Parda *et al.*, 1995). Thus, due to the production of ROS by catecholamine causes the changes of normal cellular environment in *Tetrahymena* cells and causes cell injury leading to decreased growth rate or initiation of death phase. These effects result in the ultimate depletion in the number of cells and ultimately exhibition of toxicity.

4.1.2 Initial formation of biogenic monoamines

Tetrahymena cell produce biogenic monoamines and liberate them into culture medium. Catecholamine standard solution (ClinRep®**)** was used to prepare standard chromatogram of dopamine adrenaline and noradrenaline illustrated in Fig. 4-7(a). The unknown peaks appeared in the samples were identified and detected by comparison with standard peak with respect to retention time. In biological samples, there are always some ions and trace metabolites that slightly shift the retention time of analytes but does not affect the performance of estimation. The representative catecholamines (noradrenaline, adrenaline and dopamine) were increasingly synthesized by cell and accumulated in the culture medium over time. However, the values reached maxima between 24 h and 32 h incubation. Noradrenaline was significantly formed and reached a maximum of 100 nmol after 24 h incubation. This enhanced value of noradrenaline demonstrated the exponential growth rate of the cell as depicted in Fig. 4-2 and 4-3. Dopamine and adrenaline slowly accumulated and reached to higher value of 21.7 nmol and 7.03 nmol respectively after 32 h incubation as presented in the Fig. 4-2 and 4-3. In this experiment adrenaline could not be detected until 10 h incubation and may be due to the slow accumulation in the medium or non-expression of the enzyme phenylalanine-N-methyltransferase responsible for the synthesis of adrenaline. In summary, biogenic monoamines were increasingly accumulated during exponential growth and then after gradually depleted. The depletion of monoamine synthesis could be traced back by two probable reasons; firstly the decreased growth rate of the cells and secondly the random biodegradation of the existing catecholamines in the medium. On the other hand, a higher

noradrenaline concentration was detected at the beginning of the experiments. This unexpected high concentration of noradrenaline could be obtained from the 100 µl aliquot of *Tetrahymena* culture due to pre-existing higher amount of biogenic monoamines. The monoamine in this aliquot decreased gradually due to its fast degradation and slow adaptation of cells of the new environment in 96-well plates.

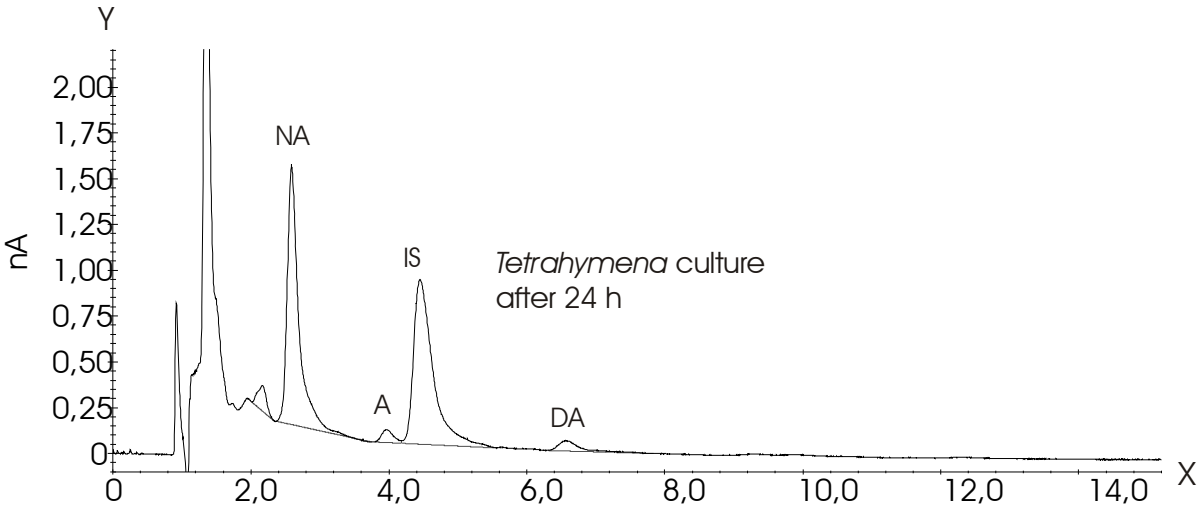


Figure 4-2: Chromatogram of catecholamine after 24 hours. Biogenic monoamines were released during cell growth.

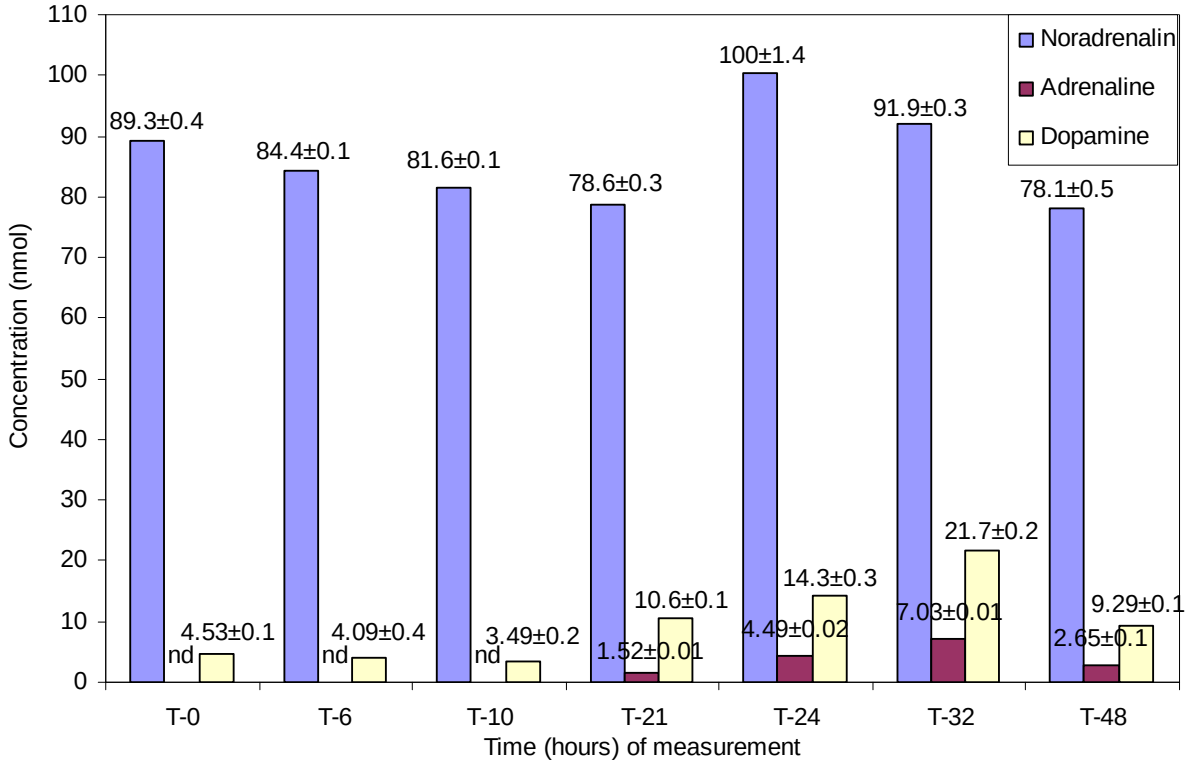


Figure 4-3: Time dependent natural formation of catecholamine (mean ± SD) in *Tetrahymena pyriformis*, where n=3.

Tetrahymena cells grow exponentially and actively synthesize biogenic monoamines. Therefore, biogenic monoamines may be a useful bio-indicator for cell proliferation and other cellular activities. This information poses the question whether a specific monoamine or all monoamines are predominantly responsible to cell proliferation activity. To address this question, dopamine, adrenaline and noradrenaline were measured in *Tetrahymena* culture at different time intervals. Wherein, noradrenaline elicited as the major dominating monoamine in culture. Thus, the appearance of higher concentration of noradrenalin may be assumed as an indicator for exponential growth of *Tetrahymena* cells. However, dopamine, noradrenaline and adrenaline concentration gradually increased over time and reached a maximum at the log phase followed by a slow depletion due to the decreasing growth rate of cells and the fast degradation in the medium.

4.1.3 Biodegradation of catecholamines

Catecholamines play a role as signalling compounds which are highly reactive and degradable in the culture medium. Many investigations reported that due to the unstable nature of the catechol ring, it is susceptible to spontaneous oxidation and conversion into quinine. This reaction is accelerated in presence of manganese or iron ions (Graham, 1978; Donaldson *et al*., 1982; Napolitano *et al*., 1995). The degradation occurred in 96-well plates in PPY medium alone and separately in *Tetrahymena* culture and was measured by HPLC. Freshly prepared standard solutions of dopamine, adrenaline and noradrenaline at nominal concentrations of 120 µg/L (0.12 ppm) equivalents to 783 nmol, 655 nmol and 709 nmol for dopamine, adrenalin and noradrenalin respectively, were also measured. They were found to be 640 nmol, 463 nmol and 396 nmol respectively. These values corresponded to recoveries of 81.7%, 70.8% and 55.8% respectively (Fig. 4-4). Due to the instability of the catechol ring almost all of the represented catecholamines were degraded, when they are incubated in the PPY-medium. After introduction of catecholamines at 120 µg/L into PPY-medium and incubation for 48 h only 60 nmol, 54.8 nmol and 15.3 nmol could be determined for noradrenaline, adrenaline and dopamine respectively as illustrated in the Fig. 4-5. Moreover some of the compounds could be converted into other catecholamines present. Hence, the degradation rates for noradrenaline, adrenaline and dopamine were found to be 85%, 88% and 97.6% respectively. The percentage of biodegradation of catecholamines was calculated with reference to measurement of standards. On the other hand, when catecholamines were added into the exponentially growing culture of cells a little replenish of degradation was found

which may be attributed to the additional synthesis of catecholamines by the cells itself as depicted in the Fig. 4-6.

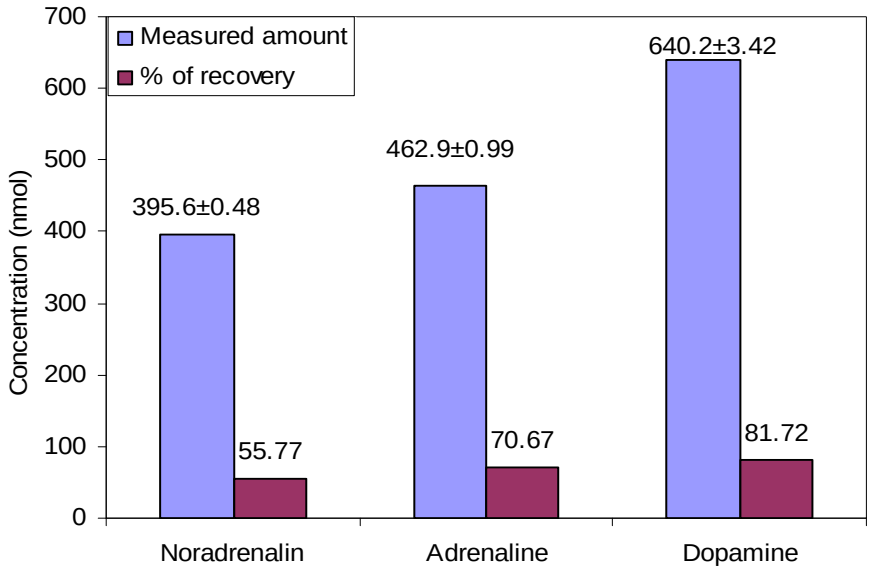


Figure 4-4: Estimation of catecholamines (mean ± SD) after solid phase extraction of fresh prepared dopamine, adrenaline and noradrenalin at concentration of 0.12 ppm that corresponds to 783 nmol, 655 nmol and 709 nmol respectively employing HPLC-ED, n=3. This experiment reveals the capacity of detection of catecholamines.

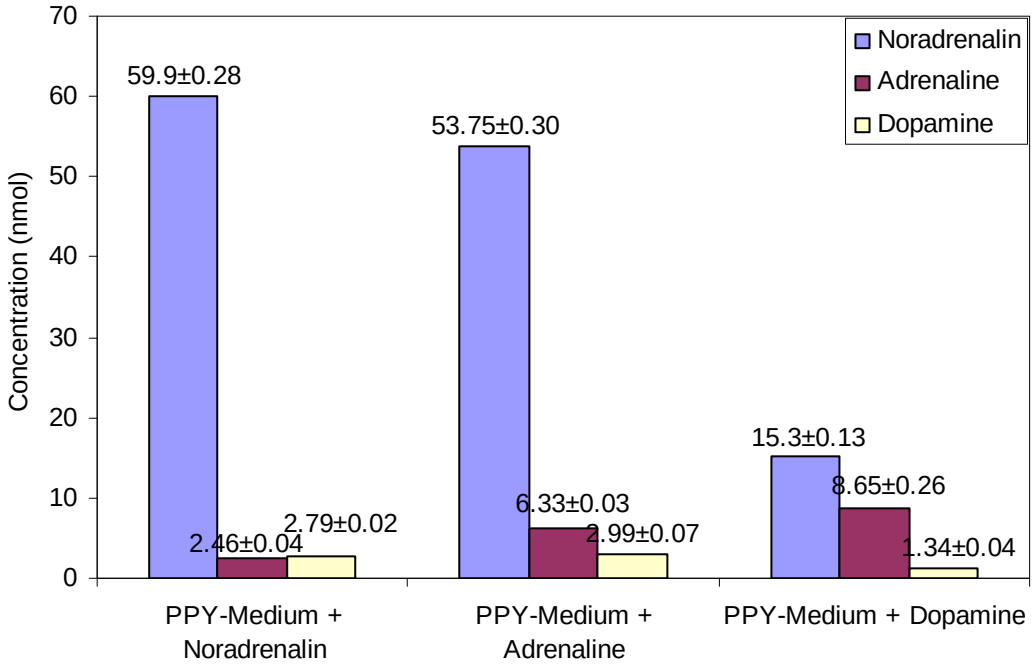


Figure 4-5: Degradation of catecholamine (mean ± SD) in the PPY-medium at concentration 0.12 ppm after 48 hours exposure, n=3.

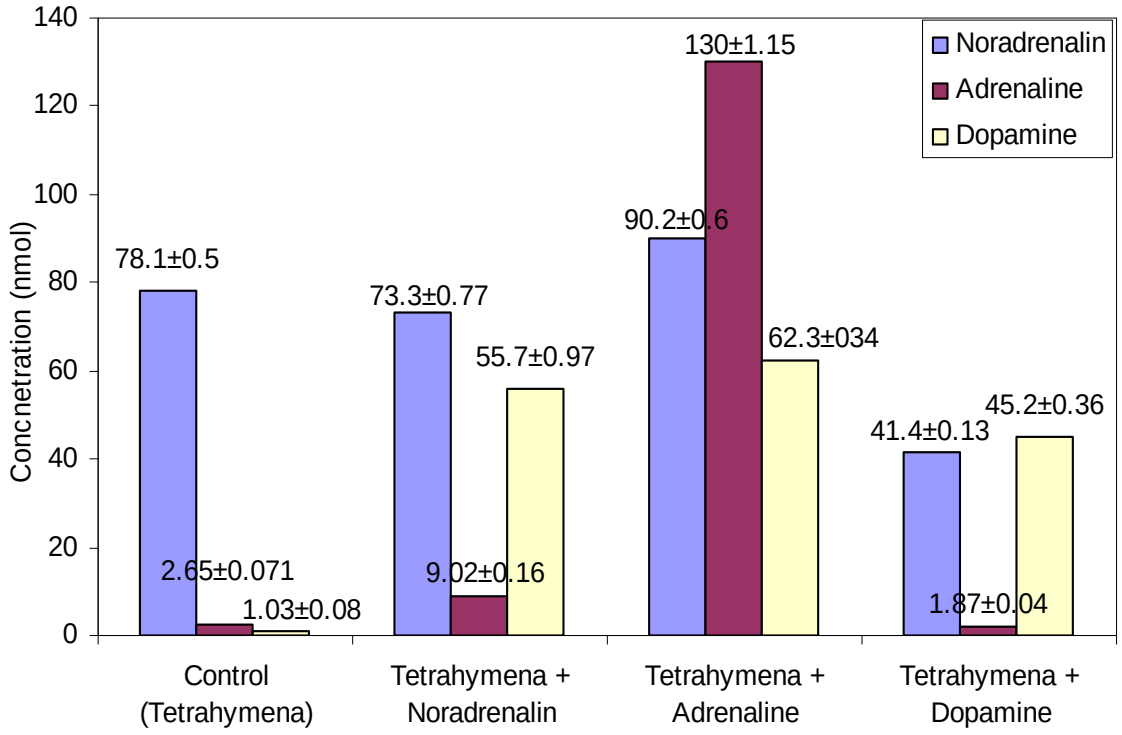


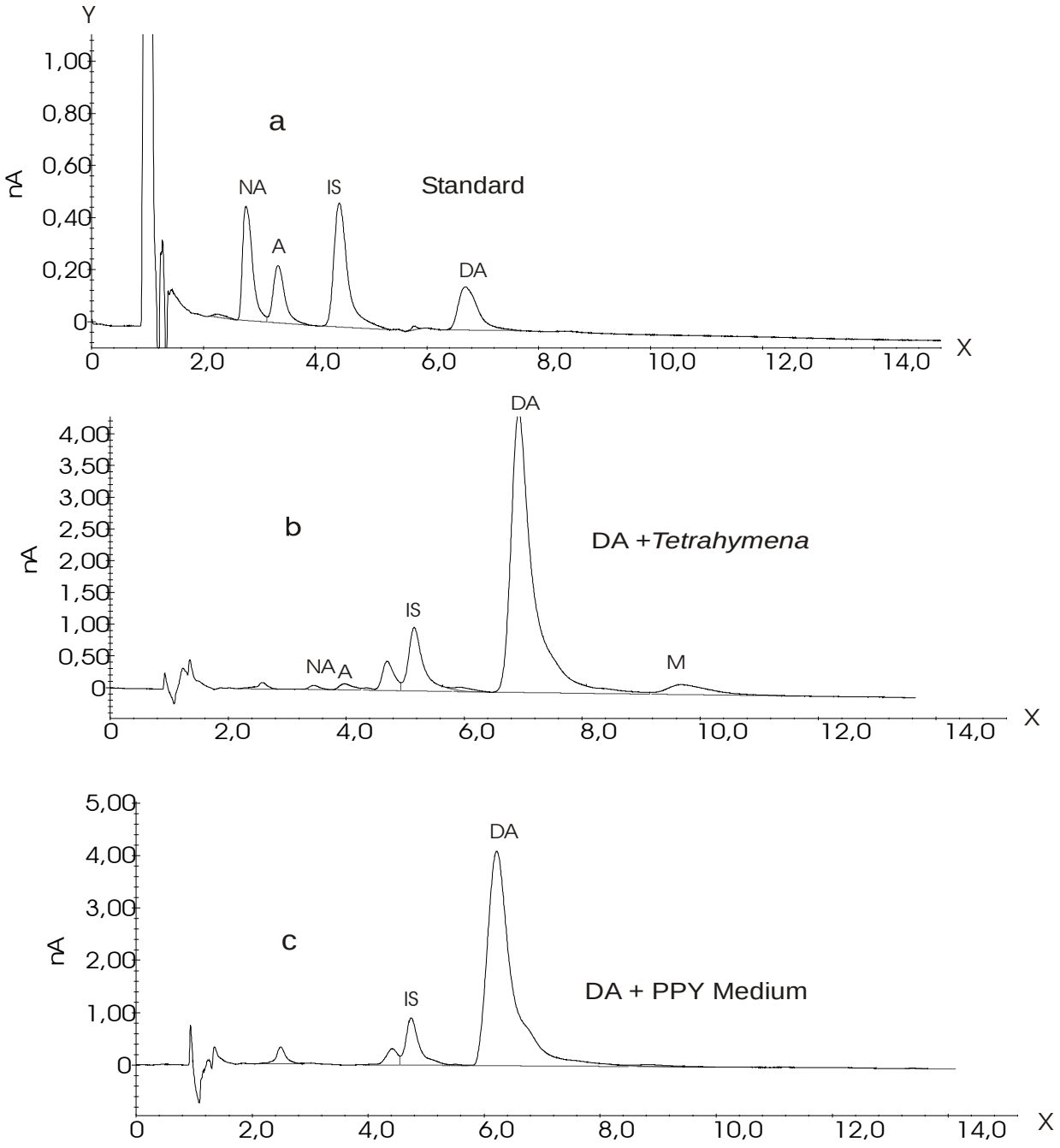
Figure 4-6: Determination of concentration and degradation of catecholamine (mean ± SD) in the *Tetrahymena* culture at exposure of dopamine, noradrenaline and adrenaline of the concentration 0.12 ppm. (Mean ± SD), n=3.

This experiment explored that the exogenous application of catecholamines strongly affected the normal synthesis of noradrenaline. It was demonstrated that the representative concentrations of catecholamines were found to be 78.1 nmol, 2.65 nmol and 1.03 nmol for noradrenaline, adrenaline and dopamine respectively in the control *Tetrahymena* cell after 48h incubation. Due to the exogenous application of catecholamines, the noradrenaline concentration depleted significantly to levels below those of control cells with the exception for adrenaline addition (Fig. 4-6). Adrenaline caused less effect to the depletion of noradrenaline. These results reflect that probably exogenous chemical pressure inhibited the enzyme dopamine β hydroxylase (DβH) responsible for noradrenaline synthesis. Especially, dopamine is more potent to inhibit this enzyme. DβH plays a key role in the catecholamine biosynthesis. Indeed, this observation indirectly correlates the higher toxic effect of dopamine with the potential interference of cell growth. Interestingly, these findings indicated the accumulation of dopamine in treated cells as compared to control cells; although dopamine is highly degradable. The dopamine accumulation may be caused by substrate inhibition of dopamine β hydroxylase and slow down the production of noradrenaline and adrenaline. Therefore, DβH could be the target enzyme of catecholamine toxicity.

4.1.4 Investigation of new detected metabolite

The 96-well plate system was used to study the catecholamine homeostasis in both normal cells and cells that were exposed to those chemicals. Since exogenous applications of catecholamines affect the reproduction of cells which consequently decreases the normal biosynthesis of catecholamines. Therefore, this effect may alter biochemical reaction in the cells leading to production of other metabolites. Following this idea, many different concentrations of all representative catecholamines were tested in *Tetrahymena* for the identification of new metabolites. The physiological concentration (0.12 ppm) for all testing compounds did not sufficiently contribute to excite and influence the cells to liberate metabolites. However, noradrenalin, adrenalin and DOPAC did not stimulate the cells to liberate metabolite even at higher concentrations used. Only dopamine and L-DOPA were attributed to the stimulation and excitation of the cells to generate and liberate a metabolite into the medium at higher concentrations as illustrated in Fig. 4-7(b). The higher concentration of dopamine was adjusted at 8 ppm. There was no product detected in dopamine exposure to PPY medium at higher concentration such as illustrated in the Fig. 4-7(c). This newly detected metabolite was relatively non-polar and eluted later in comparison to all other catecholamines tested. Also, the same experiment was conducted in presence of ascorbic acid at the concentration of 1 ppm which does not affect the formation of the metabolite as depicted in the Fig. 4-7(d). Ascorbic acid acts as cellular scavenger and plays a pivotal role to prevent ROS generation. Thus, due to the addition of ascorbic acid, it was assumed that no ROS existed in the test environments. Therefore, the detection of the metabolite in presence of ascorbic acid reveals that the production of new dopamine metabolite is biotic but not abiotic. These experiments were also extended to cell pellets and medium of exponential *Tetrahymena* culture (both native and boiled) at the same condition for dopamine exposure at higher concentration. In these cases, only cells were attributed to the generation of metabolite by influencing dopamine, but no metabolite was detected in the cell free medium even in case of boiled medium. Due to the boiling of medium of exponential growing cells, all proteins were denatured and also the number of ROS may increase. But the addition of dopamine into this medium did not produce any product as it was expected. As a result, dopamine metabolite formation is completely independent of ROS. In addition, exposure of L-DOPA was also responsible to the detection of same metabolites released by the cells as for dopamine. Probably, in the biosynthetic pathway of catecholamine, L-DOPA is converted into dopamine by the host enzyme L-aromatic amino acid decarboxylase.

Therefore dopamine is the only responsible monoamine which causes the stimulation of cells and independently triggers the generation of the metabolite.



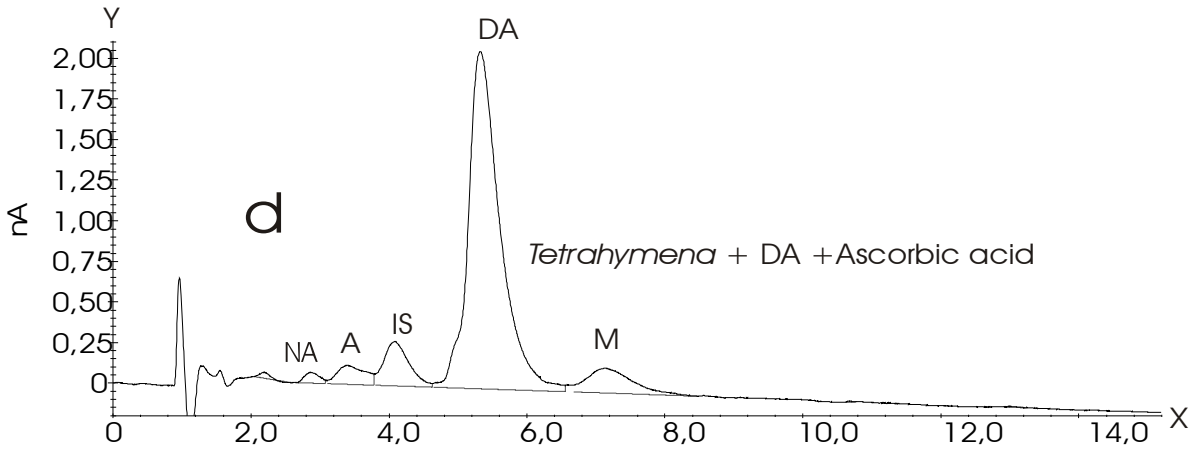


Figure 4-7: Chromatograms of catecholamine. (a) standard chromatogram of catecholamines. (b) chromatogram of dopamine exposed to *Tetrahymena* cells after 48 hours incubation where M indicates the presence of metabolites. (c) chromatogram of dopamine exposed to PPY-medium after 48 hours incubation. (d) depicts the chromatogram of dopamine exposed to *Tetrahymena* cells after 48 hours incubation. DA, NA, A and IS represent dopamine, noradrenalin, adrenalin and internal standard respectively.

It has been claimed in many publications (Alan *et al*., 1999; Graham, 1978) that dopamine produces reactive oxygen species which affect cell membranes and cellular compartments which provokes toxicity, but there was no information available on the formation of metabolites. Ascorbic acid is well documented as cellular scavenger of free radicals and its presence at physiological concentration does not affect the enzymatic reaction. So, the detection of the metabolite in presence of ascorbic acid at the same retention time might indicate that formation of the new metabolite is solely influenced by dopamine and there is no interference with ROS. However, it has been demonstrated that the generation of the metabolite truly needs the activity of cells because cell free medium of exponentially growing culture could not generate the metabolite whereas cells pellets in new medium had the ability. These findings further ascertained that the metabolite formation is truly dependent on cellular activities. It also indicated that cellular components released by the cells during growth are not responsible for the generation of the metabolite. Therefore, metabolite formation is absolutely dependent on cellular activity *in vivo* and after its formation is excreted into the medium. Dopamine probably activates unidentified enzymes in the cell. As a result those enzymes used dopamine as substrate to produce the detected metabolite.

4.2 Determination of molecular weight and formula of dopamine metabolite

4.2.1 Determination of molecular weight of dopamine metabolite employing LC/MS.

Prior the separation of metabolite and dopamine in eluents of the cells by UPLC, the sample was cleaned-up by SPE, where alumina oxide adsorbs the metabolite and other interfering substances are removed by washing. Consequently the coupling of UPLC/MS strangely contributed as sensitive tool for further separation of relevant substances in the complex mixture present. The comprehensive chromatographic conditions were chosen to optimize and resolve the interference peaks, resulting in longer retention times of the relatively hydrophilic substances. Chemical interference can be filtered out in most cases which enhance the assay selectivity. The mass chromatograms of the analytes were extracted from the total ion current (TIC) for selective detection and separation from interfering substances. The parameters were then fine-tuned by manually increasing the parameters. Most of the optimal values were similar for each analyte and as a result a generic set of conditions were adopted for the majority of the mass spectral parameters. This eliminates the need to switch between scans and aids in reducing the background signal.

The electrospray ionization of biogenic amines e.g. dopamine utilizes the primary amine moiety and the charged quaternary amine functions, respectively. The spectra for dopamine and its metabolite showed prominent ions peak as depicted in Fig. 4-8. The ion that selected the dopamine and metabolite was abundant. Extracted mass chromatograms of the analytes showed peaks at retention time (RT) of 4.4 and 5.8 minand represent molecular masses of $[M+H^+]$ = 154.087 and $[M+H^+]$ = 151.1 respectively. The molecular weights of 154.087 and 151.1 correspond to dopamine and metabolite respectively. The mother compound dopamine (154 *m*/z) and its metabolic product (151 *m/z*) were determined by full scan. The intensity and pattern of the $M+H^+$ ions were very sensitive to the collision energy due to the existence of amine and hydroxyl groups. The collision energy was optimized to obtain the most predominant fragment ion with the precursor reduced by more than 95% of its original abundance. The mass spectrometric specificity of the analyte was confirmed by the absence of collision-cell ‘crosstalk’ peaks observed in the mass chromatography when a standard containing individual compound was assayed. Although enhanced chromatographic resolution could potentially improve selectivity by reducing competitive ionization from co-eluting material, the LC/MS parameters described appear sufficient for this assay. Moreover, the separation of dopamine and metabolite by UPLC and consequently ionization and mass spectrometric detection could be performed in similar elution order as with HPLC-ED. In both cases dopamine eluted earlier than the metabolite indicating that the metabolite is less polar than dopamine.

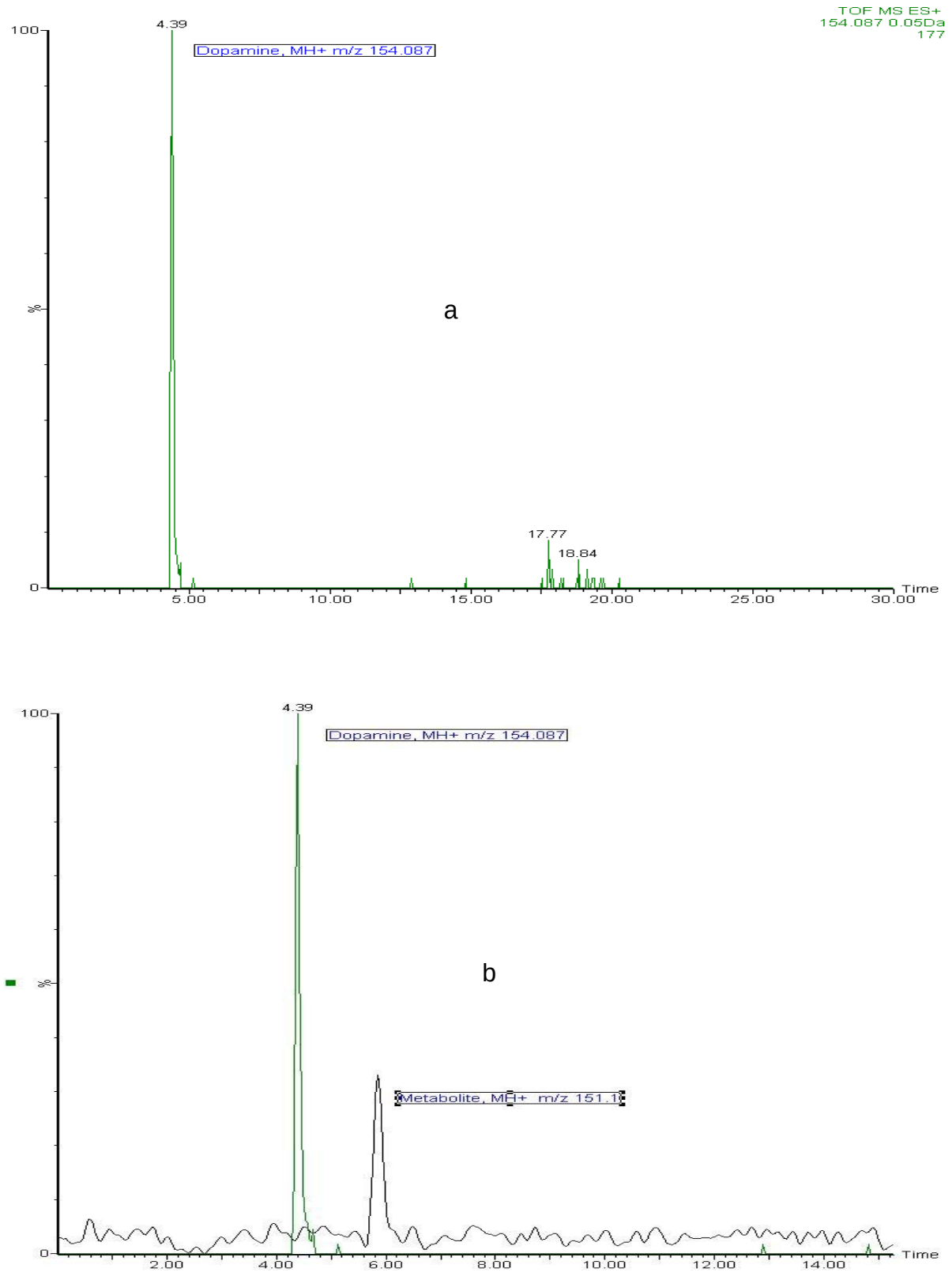


Figure 4-8: ESI/LC/MS spectrum of dopamine (m/z 154) and metabolite (m/z 151). Where, figure (a) and (b) represent the mass spectrum of standard dopamine solution, and metabolite and dopamine in the cell extract respectively.

To sum up, a liquid chromatographic-mass spectrometric method has been developed and validated for the determination of dopamine and its metabolites in *Tetrahymena cell* extracts using an aluminum oxide solid-phase extraction (SPE) procedure for sample clean up prior to ESI/LC/MS analysis. This method is simple, rapid, accurate and suitable for the analysis of these analytes in plasma or other biological fluid samples from clinical, preclinical, or pharmacological studies. One major advantage of aluminum oxide extraction using robotic liquid handling and a 96-well sample cluster in conjunction with ESI/LC/MS was to speed up the sample preparation. Preparation time was reduced significantly compared to manual aluminum oxide extraction with roughly a four-fold improvement in sample throughput realization. Small volumes of elution solution (1µl) allowed direct injection without evaporation and reconstitution. The retention time was shortened significantly due to the minimized interference from sample matrix when utilizing tandem mass spectrometry. In addition to the considerable saving in labor and time, the costs associated with the clean up procedure in this assay were much less expensive. Development of this method has been successfully applied in determining the molecular weight of a so far unknown metabolite.

4.2.2 Measurement of accurate mass and derivation of molecular formulae of dopamine metabolite employing FTICR/MS

High-field (12 Tesla) Fourier-transform ion-cyclotron resonance mass spectrometry (FTICR-MS) has many prominent characteristics, such as its ultrahigh resolution in broadband measurements and its high mass accuracy (routinely better than 200 ppb with an internal standard). The accurate masses $[M+H]^+$ from dopamine and its metabolite were shown in Table 4-2 together with the calculated empirical formulae. All peaks in the spectrum were below m/z 200.

Table 4-2: Data analysis from FT-ICR MS for dopamine and metabolite liberated by *Tetrahymena.*

m/z	Measured mass		Error (ppm)	Comments
	LC/MS	FT-ICR/MS		
154	154.087	154.08632	0.01	Dopamine
151	151.10	151.08659	0.01	Possible metabolite
		151.09649	0.00	Impurities
		151.11174	0.03	Impurities
		151.07536	0.03	Impurities

Due to the huge peak capacity of FTICR mass spectrometry, FT mass spectra provide the most convincing direct experimental evidence for the extraordinary molecular diversity of

complex materials present. In these, the molecular-level intricacy of the complex unknowns is most adequately converted into very highly resolved and, consequently, extremely information-rich signatures. Mass accuracy power of FT/MS clearly demonstrated the mass and molecular formula of dopamine and metabolite (Fig. 4-9 and 4-10). Many different molecular formulae at same mass range were found. Four peaks were considered for the assignment of the real peak of the metabolite such as m/z 151.08632, m/z 151.09649, m/z 151.11174 and m/z 151.07536 leading to the empirical formula $[M+H^+]$ of X? (Confidential), $C_6H_{15}O_4$, $C_{10}H_{15}O$ and $C_9H_{11}O_2$ respectively.

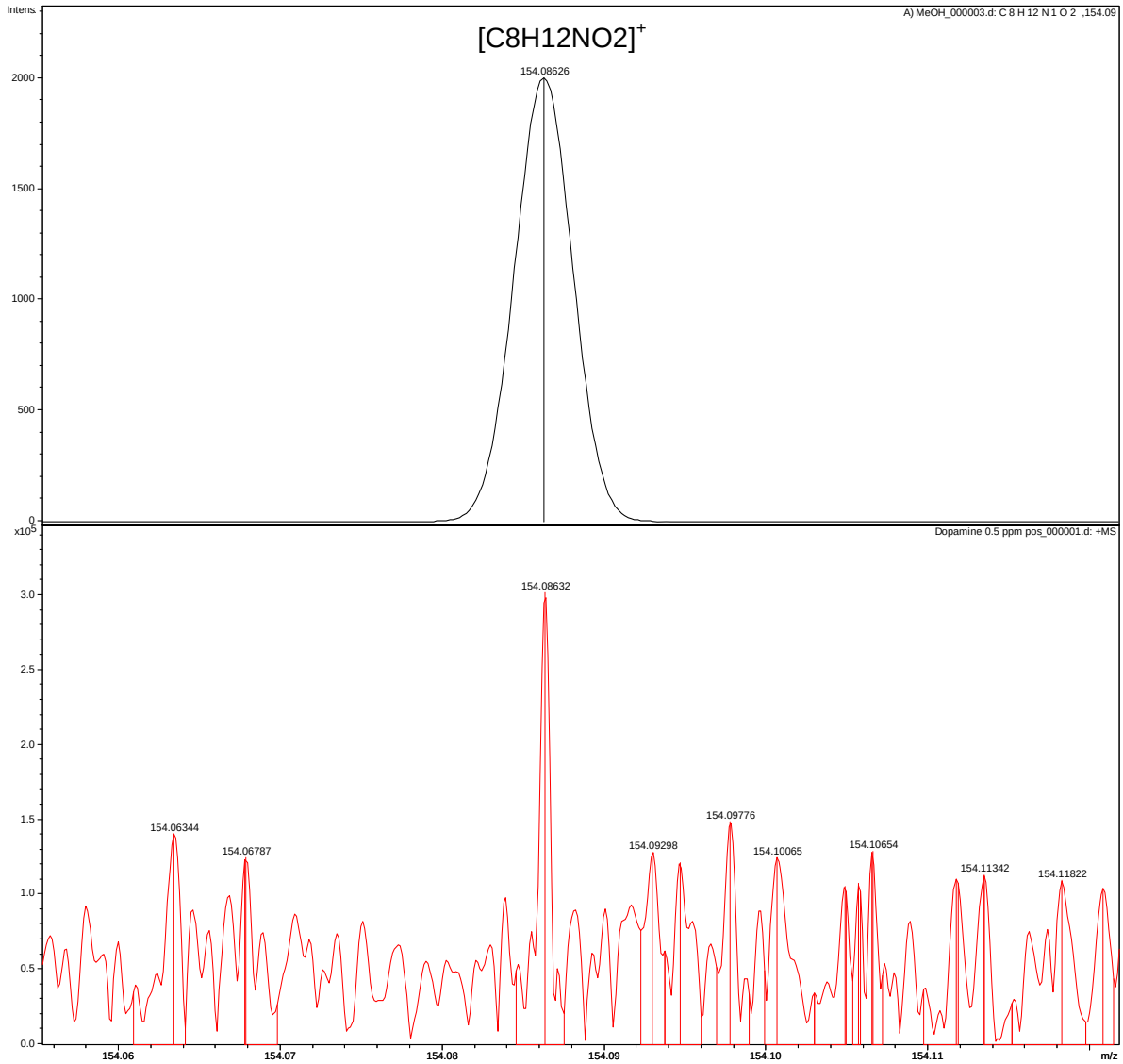


Figure 4-9: FT-ICR mass spectrum of standard dopamine.

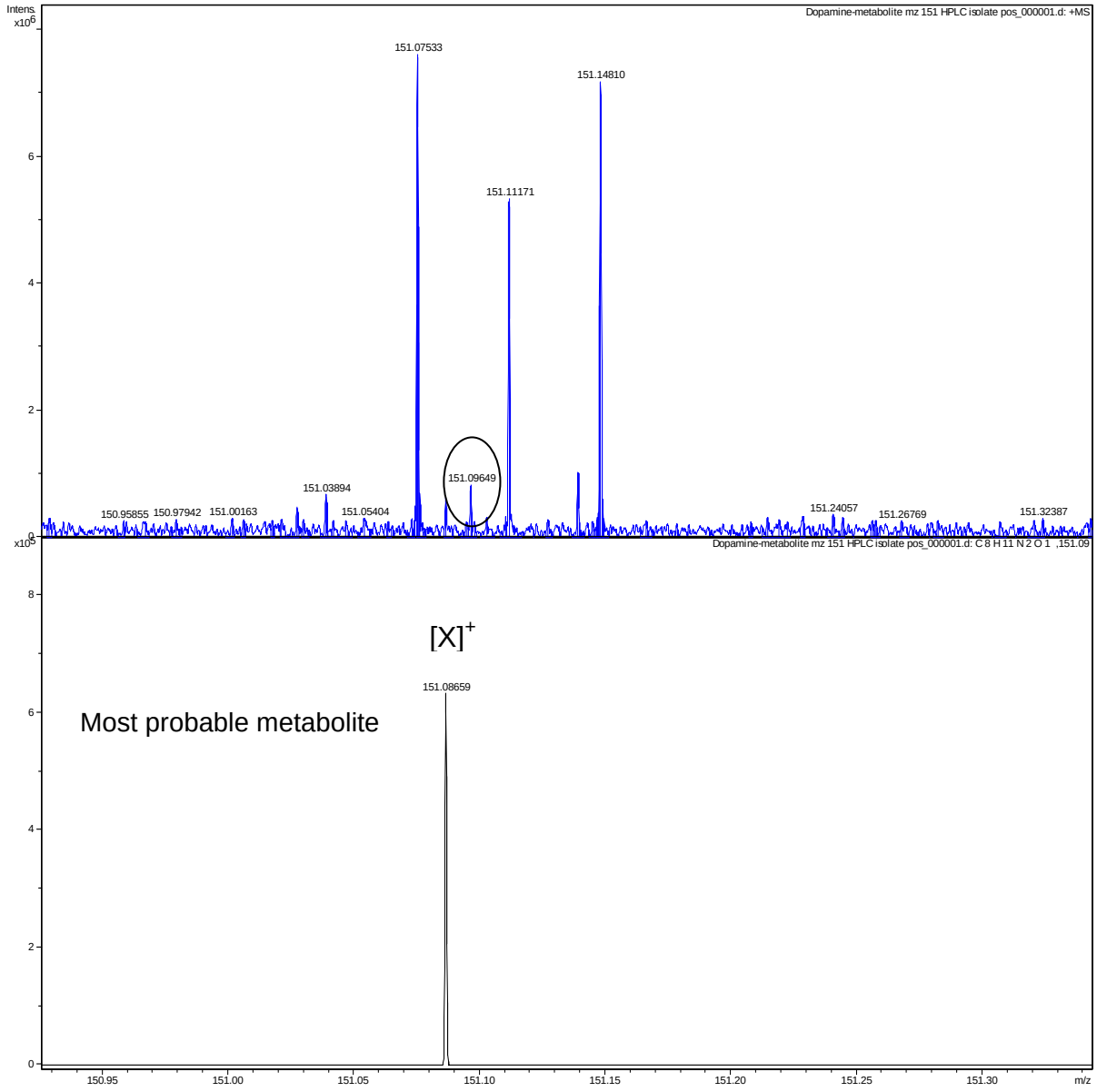


Figure 4-10: FT-ICR mass spectrum of metabolite in the cell extract. A number of peaks appear in the mass spectrum. But $[X]^+$ would indicate the most possible elemental composition of metabolite.

The close observation of molecular formula and structure of all catecholamines and their metabolites suggest that the possible elemental composition of the metabolite should be X. Supporting, the formula of the metabolite conserves the structural similarity of dopamine due to the presence of same number of carbon atoms and the benzene ring. On the other hand, $C_6H_{14}O_4$, $C_{10}H_{14}O$ and $C_9H_{10}O_2$ did not match the structural similarity of dopamine. These three compounds do not contain the same number of carbon atoms of dopamine and structural similarity. Therefore, due to the dissimilarity of structure and carbon atoms these three formulas clearly represent the presence of impurities. In contrast the formula X reasonably

supports the possible structure of the metabolite. The transition of element might be compromised by the creation of a new molecular functionality

4.3 Identification of dopamine receptor in *Tetrahymena thermophila*

Many high affinity fluorescent rhodamine derivatized ligands for D1 and D2 receptor have been developed. Rhodamine SKF-38393 agonist and rhodamine SCH-23390 antagonist fluorescent probes demonstrated faithfully the cellular localization of the D1 dopamine receptor subtype. The photographic representation of ligands binding to the D1 receptor was investigated in *T. thermophila*. The Fig. 4-11 depicted the photograph of control cells under fluorescent microcopy. Control cell did not exhibit fluorescent signals in our experimental setup.

However, the exposure of fluorescent agonist SKF-38393 in the cells emitted fluorescent signals. Cell incubation was carried out for many different times. The maximum binding of the agonist was revealed after 3 h incubation which causes saturation binding of receptor. Five molar concentrations; 5 μM, 10 μM, 20 μM, 40 μM and 80 μM of ligand were used for this study. Fluorescent photograph of cells was depicted in the Fig. 4-12. However, longer time exposure of ligands over 6 hours again caused the decrease of receptor binding as well as decrease of fluorescent signals. The specific binding of the agonist to D1 receptor was confirmed with the addition of antagonist SCH-38393. Both ligands are specific and conserve higher binding affinity to D1 receptors. They were added together at the same concentration. The photograph of cell after the exposure of agonist and antagonist revealed no binding of agonist such as illustrated in Fig. 4-13. Thus, the affinities were encountered by each other. This is caused by the addition of same molar concentration of agonist and antagonist in the cell due to competitive binding to the D1 receptor. Additionally, another experiment was carried out for agonist SKF-38393 with non-labelled antagonist (±) butaclamol. These ligands were added to the same five concentrations mentioned. After the addition of butaclamol, the cell did not show fluorescence signals; results are presented in Fig. 4-14. It was already discussed that the two types of dopamine receptors exists in dopaminergic cells. Therefore, the assessment of cross-affinity of agonist SKF-38393 to D2 receptor is of important concern. For this reason, we have selected the D2 specific receptor antagonist spiperone. SKF-38393 and spiperone were added at once and exposed in the same way as before. Spiperone did not inhibit SKF-38393 binding to the D1 receptor (Fig. 4-15). Furthermore, cells were treated

with 2 ppm L-DOPA followed by 24 h incubation. These pretreated cells were again used for the experiment of SKF-38393 at same condition. This caused no significant difference in fluorescent signals as compared to ligands added to the cells without treatment with L-DOPA (Fig. 4-16).

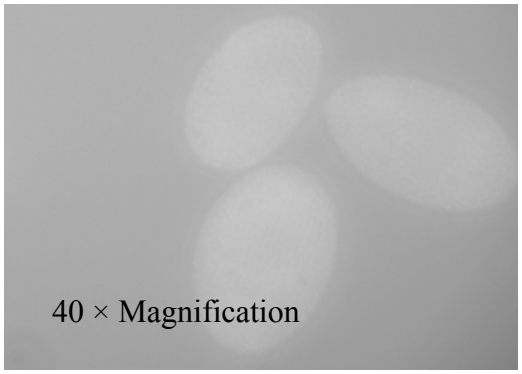


Figure 4-11: Control *Tetrahymena thermophila* cell.

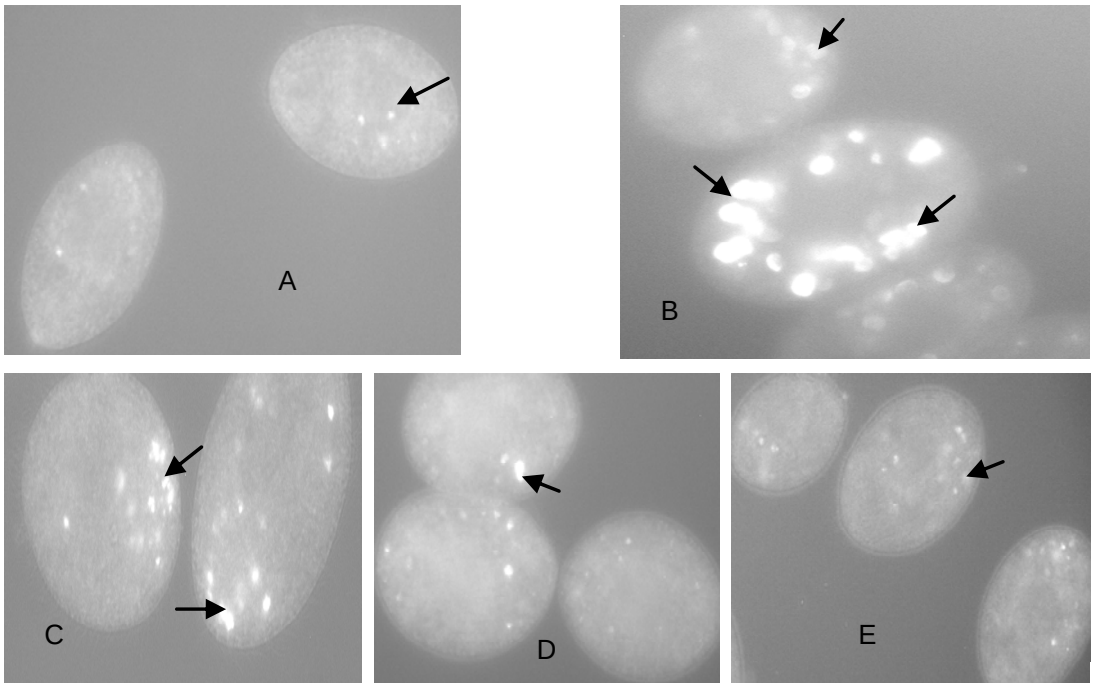


Figure 4-12: Fluorescence localization of D1 type dopamine receptor in *Tetrahymena thermophila*. Fluorescent agonist probe, rhodamine- SKF-38393 bind specifically to D1 receptor. This receptor is internalized into endosome due o the sensitivity of SKF-38393. The entire photograph shows total binding of receptor after 3 hours exposure of agonist. This time period causes saturation binding of ligands and reveals stronger signals. The figures A, B, C, D and E represents 5 µM, 10 µM, 20 µM, 40 µM and 80 µM concentration of ligands respectively.

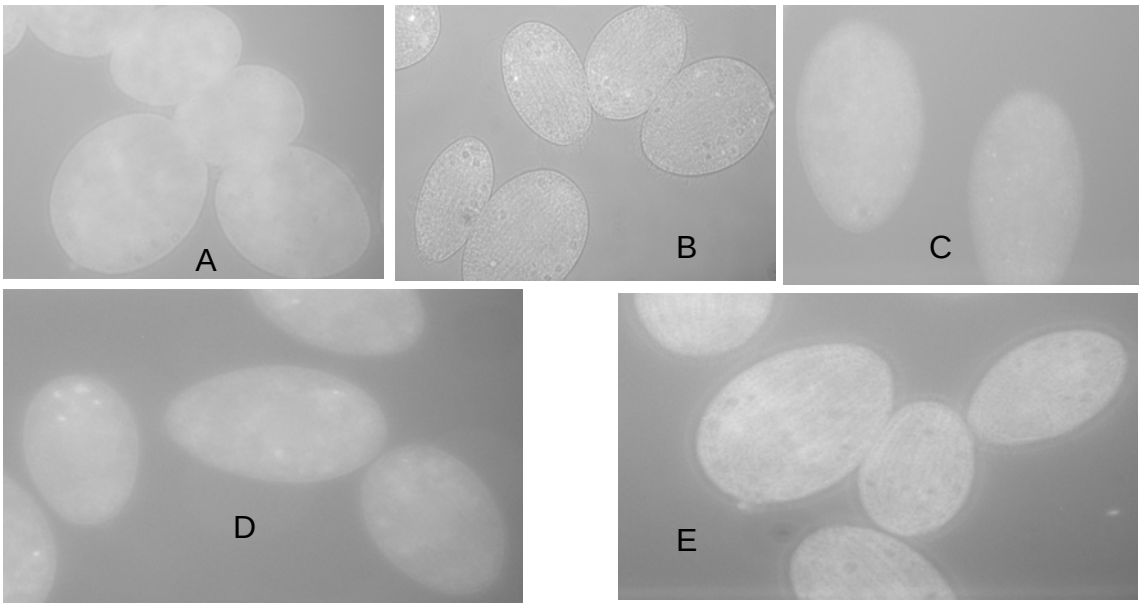


Figure 4-13: Experiment for specific binding of agonist SKF-38393 to D1 receptor in the *Tetrahymena* cell. The agonist, SKF-38393 and D1 specific fluorescent probe antagonist SCH-23390 were applied together to the cells at equal five different nominal concentrations at same incubation time. Both ligands undergo binding competition to D1 type receptor in cell which does not show fluorescence signals. Due to the higher affinity of agonist to antagonist than receptor causes neither agonist nor antagonist bind to D1 receptor. Thus clearly reveals the specific binding of SKF-38393 to D1 receptor. The figures A, B, C, D and E represent the ligand concentrations of 5 μM, 10 μM, 20 μM, 40 μM and 80 μM, respectively.

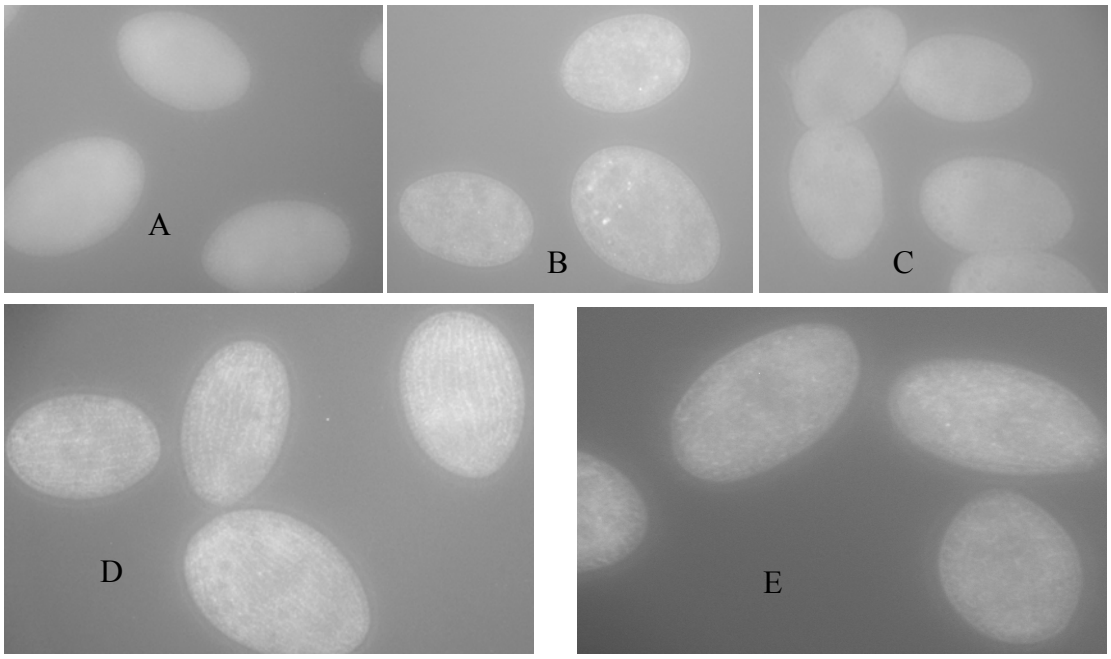


Figure 4-14: Assay of specific binding of agonist SKF-38393 to D1 type receptor in the *Tetrahymena*. It was studied with the addition of non fluorescent dopamine receptor antagonist (+)-butaclamol same as before. This study exhibited the same result of Fig.3. The figures A, B, C, D and E represent the ligand concentrations of 5 μM, 10 μM, 20 μM, 40 μM and 80 μM, respectively.

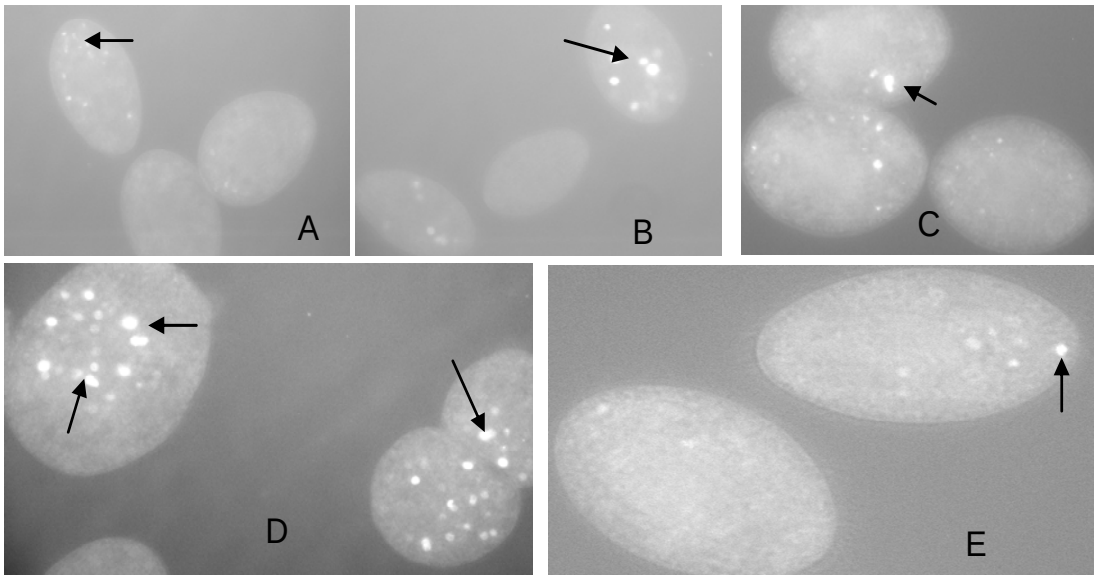


Figure 4-15: Assay for the cross binding of SKF-38393 to D2 type receptors. SKF-38393 and non-fluorescent D2 specific antagonist, spiperone were added together to the cells at the equal five nominal concentrations and incubated for 3 hours. In this study spiperone can not inhibit SKF-38393 to bind D1 receptor. However, spiperone bind only to D2 type receptors and competes with D2 specific agonist. Thus, indicates that SKF-38393 agonist has no cross affinity to D2 type receptor. It is truly bound to D1 receptors. The figures A, B, C, D and E represent the ligand concentrations of 5 µM, 10 µM, 20 µM, 40 µM and 80 µM, respectively.

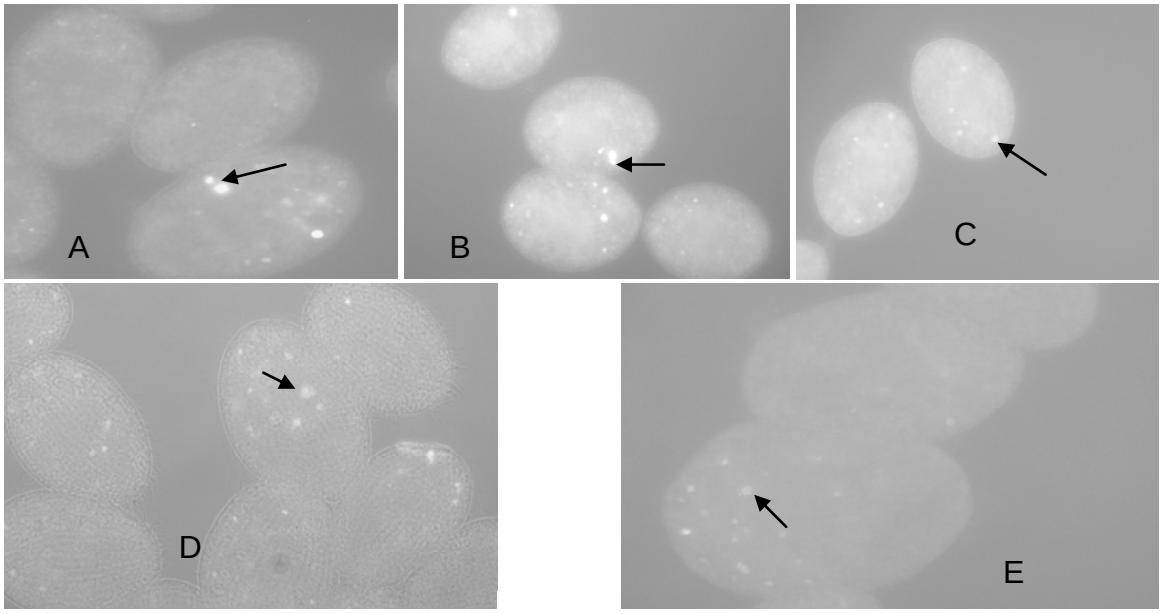


Figure 4-16: *Tetrahymena* cells were treated with 2 ppm L-DOPA for the stimulation of dopamine receptor over 24 h incubation. These treated cells were used for an assay of agonist SKF-38393 binding to D1 specific receptor. Ligand was added as before. In this case, cell exhibited fluorescence signal upon agonist binding to D1 receptor. Due to the illustration of weaker signal as compared to Fig. 2, revealed that the L-DOPA could not stimulate protein expression of dopamine receptor. Here A, B, C, D and E represent the ligand concentrations of 5µM, 10 µM, 20µM, 40µM and 80µM, respectively.

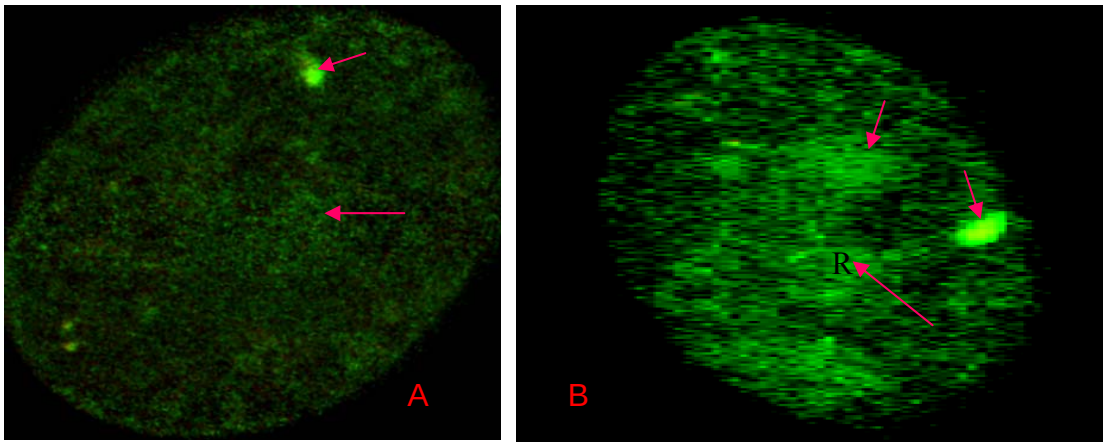


Figure 4-17: *Tetrahymena* cell was exposed by SKF-38393, and figures are captured by LSM. Figure A and B represent the normal and transparent picture of same cell. The transparent picture indicated that D1 receptor is abundant in the endosome and rough ER rather than cell membrane. This receptor is internalized in the endosome and may be activated by the agonist stimulation. R indicated the ribosomal location of D1 type receptor.

Therefore, the fluorescent ligand binding assay clearly demonstrated the existence of D1 type receptor in *T. thermophila* cell. In addition, the location of this receptor was analyzed by capturing photograph by means of laser scanning microscope (LSM). Only one suitable concentration, 40 µM of SKF-38393 was used for this study. The transparent picture (Fig. 4-17) of the cell exhibited a brighter signal in the cytoplasm than in the membrane site. Therefore, it is revealed that the receptor is densely organized and located in the cytosol. This photograph also showed that then D1 receptors are abundant in endosome and rough endoplasmic reticulum (ER). Rough ER remains bound to the ribosome where the proteins are synthesized and migrated to Golgi bodies through transport vesicule.

The fluorescent agonist SKF-38393 binds specifically to dopamine D1 (D1/D5) type receptors in *Tetrahymena thermophila*. It's binding to the receptor increases with the exposure time up to 3 h. Therefore, this time period causes saturation of receptor binding as well as increases the fluorescence signal. However, longer exposure time of ligands over 6 h causes desensitization of receptors and consequently reduction of fluorescence signal was found. Prolonged or repeated exposure of ligands often decreases the responsiveness of the receptor and alters affinity of this ligand (Sun *et al.*, 2003). Five different nominal concentrations of the agonist were applied for binding assay to the D1 receptor wherein 20 µM and 40 µM concentrations of SKF-38393 displayed bright signals. The affinity of agonist binding to the D1 receptors was blocked or damped due to the use of antagonist SCH-23390 at same molar concentrations. Both ligands undergo binding competition to the D1 receptor

and antagonize their affinity by each other. The same result was found when SKF-38393 and SCH-23390 were used together (Ongini *et al*., 1985; Furukawa and Morishita, 1997; Zhu *et al*., 2000). Due to the higher affinity of agonist to antagonist than to D1 receptor neither binding of agonist nor antagonist to the cell receptor is caused. Furthermore, the cells were exposed to the labelled agonist SKF-38393 and the non-labelled common dopamine receptor antagonist (+) butaclamol at same molar concentrations. Butaclamol acts as universal antagonist for dopamine receptors (Pugsley *et al*., 1976). It also revealed the same result as for SCH-23390. However, a hypothesis claimed that the antagonist of one type dopamine receptor could not prevent the binding of another type dopamine agonist (Furukawa and Morishita, 1997; Zhu *et al*., 2000; Birrell *et al*., 2002; Ferrari *et al*., 2004). So, the D2 specific receptor antagonist spiperone was used to resolve the dispute of cross-binding of SKF-38393 agonist other than D1 receptor. Spiperone is a non fluorescent ligand and bind to D2 receptor (Amenta *et al*., 1999). The exposure of spiperone could not inhibit/antagonize the binding of D1 specific agonist SKF-38393 while SCH-23390 inhibits binding of SKF-38393 to D1 receptor. The study of SKF-38393 and spiperone causes binding of SKF-38393 to D1 receptor which still develops a fluorescent signal. These finding suggests the specific binding of SKF-38393 to the D1 receptor. L-DOPA stimulates the expression of dopamine receptor in mammals (Trugman *et al*., 1991). However, in contrast to mammals L-DOPA could not stimulate dopamine receptors in *Tetrahymena* cells.

The G protein coupled receptor (GPCR) mediates the role of D1 receptors. According to the current ideas about GPCR trafficking in general (Dohlman *et al*., 1991), those dopamine receptors in particular are internalized in early endosome from which they may be recycled to the plasma membrane or targeted for turnover in late endosome (Ng *et al*., 1994). A number of transfection studies of D1 and D2 have shown a predominantly cytoplasmic localization for D2 (Ng *et al*., 1994; Vickery and von Zastrow, 1999; Prou *et al*., 2001). Surprisingly, agonist stimulation, results in internalization of D1 (Ng *et al*., 1994; Vickery and von Zastrow, 1999). Internalization of the receptor following ligands binding is known to occur for a number of GPCRs, including the D1 type receptors, and this leads to a lowering of surface receptor density (Ferguson, 2001; Sun *et al*., 2003). Same observation was found in the photography of LSM which is discussed in this study. It was clearly revealed that the D1 receptor protein is abundant in the cytosol whereas few receptors are positioned at the cell membrane site (Fig. 4-22). Probably the SKF-38393 exposure causes D1 receptor internalization into the endosome. Recent literature reported the location of dopamine receptors in endosome in

cytoplasm (Elizabeth *et al*., 2007). However, our observation also indicated that this receptor is visible in other areas of the cell like rough ER. This cell organelle contains ribosome which produces all kind of proteins including D1 receptor protein. The observation claimed that D1 receptor is also present around the rough ER in addition with transport vesicule.

4.4 Toxicity and genobiotic transfer of 5-fluorouracil in the aquatic environment

4.4.1 5-Fluorouracil toxicity to *S. vacuolatus* and *T. pyriformis*

Cell growth inhibition to 5-FU was determined by the use of nominal concentration and described by the symmetric logistic sigmoid model. A concentration response curve was constructed with the applied concentration of compounds versus growth inhibition of cells. The number of algae cells was directly proportional to the optical density which indicates that increase of cell number corresponds to increase of OD. Percentage of cell growth inhibition was determined with respect to control. This study revealed the acute toxicity of 5-FU to algae cells. However, no significant cell growth inhibition was found at low concentrations (6.25 ppm or below) for both exposure times whereas all other applied concentrations exhibited significant cell growth inhibition (Table 4-3). The effective concentration (EC) of 5-FU was calculated by plotting the percentage of growth inhibition versus concentration of 5-FU (Fig. 4-11). EC20 for 24 h and 48 h exposure were estimated to 30.2 and 38.9 ppm respectively. EC50 for 24 h exposure of 5-FU was found to be 159.9 ppm. When algae cell incubation continued for 48 h EC50 was not observed at the 50% intercept of the regression curve (Fig. 4-18).

Table 4-3: Cell growth inhibition of microalgae, *Scenedesmus vacuolatus* (n = 3) with exposure of 5-fluorouracil.

Treatment	Concentration of 5-FU (ppm)	% of cell growth inhibition after 24 h ± SD	% of cell growth inhibition after 48 h ± SD
Algae (control)	0	0	0
Algae + 5-FU	6.25	n.d	n.d
Algae + 5-FU	12.5	2.86 ± 0.54	n.d
Algae + 5-FU	25	14.3 ± 1.9	3.30 ± 0.5
Algae + 5-FU	50	20 ± 3.8	16.5 ± 2.5
Algae + 5-FU	100	42.9 ± 5.02	26.4 ± 3.7
Algae + 5-FU	200	54.3 ± 7.89	27.5 ± 4.0

Note: n.d represented as not determinable.

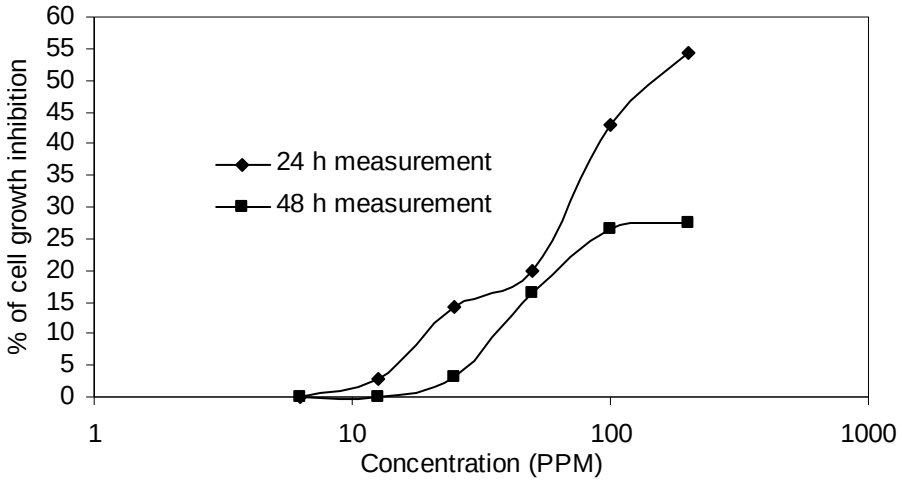


Figure 4-18. Concentration-response curve of 5-FU in microalgae, *Scenedesmus vacuolatus.* Cells were exposed for 24 hours, 48 hours and 72 hours respectively. Data points represent mean values of three replicates. 5-FU concentration was expressed by a logarithmic scale.

On the other hand, acute toxicity was also exerted on *Tetrahymena* at the introduction of 5-FU which resembles toxicity to algae. But *Tetrahymena* cells presented more sensitivity to 5-FU as compared to algae. Comparatively low concentrations of 5-FU were associated with large growth inhibition in *Tetrahymena*. That growth inhibition was ascertained by measuring optical density of cells after 24 h and 48 h incubation respectively. Every treatment of 5-FU was evoked acute toxicity (Table 4-4). The higher applied concentrations 5-FU (7.81 ppm or more) were not considered for calculation because at these concentrations showed almost the same effect as at 7.81 ppm. A concentration response curve was drawn by plotting percentage of cell growth inhibition versus concentration which clearly implied EC50 for both exposures times. EC50 values for 24 h and 48 h were demonstrated to be 1.23 ppm and 1.02 ppm respectively (Fig. 4-19).

Table 4-4: Cell growth inhibition of *Tetrahymena pyriformis* (n = 4) with exposure of 5-fluorouracil.

Treatment	Concentration of 5-FU (ppm)	% of cell growth inhibition after 24 h ± SD	% of cell growth inhibition after 48 h ± SD
Tetrahymena (control)	0	0	0
Tetrahymena + 5-FU	0.24	1.8 ± 0.002	n.d
Tetrahymena + 5-FU	0.48	22.98 ± 0.001	23.01 ± 0.004
Tetrahymena + 5-FU	0.97	44.98 ± 0.002	68.18 ± 0.004
Tetrahymena + 5-FU	1.95	61.71 ± 0.001	88.49 ± 0.010
Tetrahymena + 5-FU	3.91	74.03 ± 0.002	91.9 ± 0.080
Tetrahymena + 5-FU	7.81	77.99 ± 0.001	92.19 ± 0.018

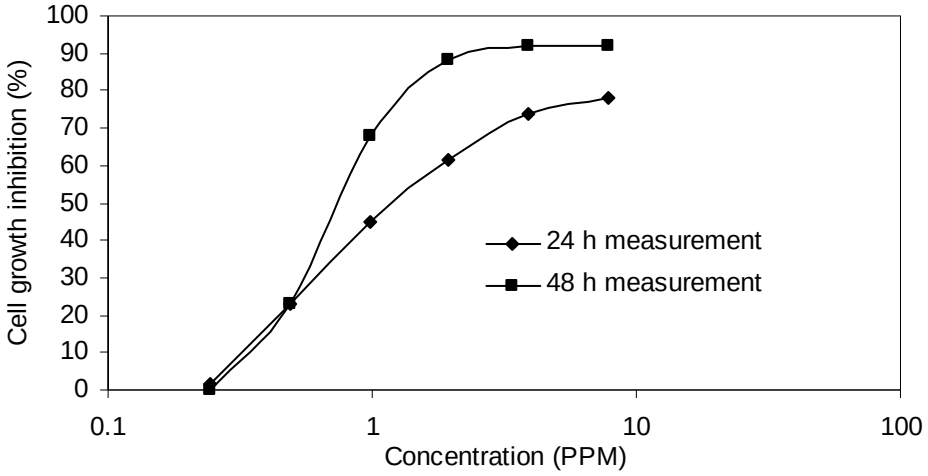


Figure 4-19. Concentration-response curve of 5-FU in protozoan, *Tetrahymena pyriformis* after 24 hours and 48 hours exposure respectively, n=4. 5-FU concentrations are expressed by a logarithmic scale.

Microalgae and ciliate protozoa play a role as bio-accumulating factors in the aquatic environment. The observed cell growth inhibition due to the exposure of these cells to 5-FU initiate the death phase. 5-FU causes severe toxicity to dihydropyrimidine dehydrogenase (DPD) enzyme deficient patients (Boisdron-Celle *et al.,* 2007, André and van Kuilenburg, 2004) and cytotoxicity to the cells. Therefore, its frequent discharge into the aquatic environment makes it a pseudo-persistent substance and affects microbiota. To a low extend 5-fluorouracil enters into the microalgae probably via the lipophilic interaction and consequently accumulates in DNA and RNA that results in the repetitive insertion and excision of genetic materials (Ingraham *et al*., 1982; Kufe *et al*., 1983). DNA which incorporates 5-FU constrained small fragmentation of DNA especially at higher concentrations of 5-FU and this substitution of DNA obliged to a slow rate of elongation of DNA strands during its synthesis (Scheutz and Diasio, 1985). Each of these aspects of 5-FU incorporation may contribute to the cytotoxicity of the compound and might appear via affecting the cell growth inhibition of organisms tested. Indeed, 5-FU is degraded by dihydropyrimidine dehydrogenase (DPD) enzyme. So the cell may cause the degradation of a small percentage of incorporated 5-FU which does not contribute to the toxicity against the cell system. Furthermore, 5-FU concentration in the culture medium continually declined due to its fast uptake during reproduction of cells. The cells take up 5-FU from the medium and are incorporated into the genome in addition with excreted small percentages via enzymatic degradation. Therefore, the net concentration of the compound apparently decreased during exposure time. Although 5-FU depleted in the medium it causes nevertheless acute toxicity in both algae and protozoans. The actual concentration of 5-FU in the test medium of algae and

protozoans was not measured during exposure time. Thus, the effective concentration may be overestimated because of depletion and potential lower existing concentrations of 5-FU in the test. Moreover, the toxicity test may not be fully reliable because the depletion rate and existing concentration of 5-FU was not measured throughout the algae and protozoan culture medium.

4.4.2 Bioaccumulation of 5-fluorouracil in microalgae

The bioaccumulation of 5-FU in microalgae *Scenedesmus vacuolatus* was measured via exposure of radio-labelled [2-^{14}C] 5-FU over 24 h. The untreated control algae cells were found at base level radioactivity by counting with a scintillation counter which was considered as zero activity. However, cell pellets of treated algae from 200 mL culture yielded 25,090 DPM activity equivalents to 27.76 ng of labelled 5-FU. However, culture medium retained a smaller percentage of 5-FU. The un-accumulated 5-FU was exhibited 13,660 DPM which is equivalent to 15.11ng of labelled 5-FU (Table 4-5). Therefore, the bioaccumulation factor (BAF) of 5-FU was derived as 18.4×10^3 according to equation (5). So, cell pellets and medium together were found to show 38760 DPM activity equivalents to 17.46 nCi which corresponds to 77% recovery.

Table 4-5: Determination of bioaccumulation of 5-fluorouracil applying radio-labelled [2-14C] 5- fluorouracil in *Scenedesmus vacuolatus* at 24 hours exposure whereas n=3

Name of experiment		Radioactivity (DPM) ± SD	Radioactivity in 200 mL culture.	Amount of [2-^{14}C]5-FU	Bioaccumulation factor (BAF)
Control algae	Cell pellet of 20ml	0	0	0	0
	Supernatant of 5 mL	0	0	0	
Algae + 5-FU	Cell pellet of 20ml	2509 ± 253	25090	27.76 ng	18.4×10^3
	Supernatant of 5 mL	341.5 ± 33	13660	15.11 ng	

Note: The weight of 10 ml algal suspension is 1 mg.

5-FU is actively accumulated in algae cells, perhaps partly through lipophilic interaction. It is inserted into the genetic material of algae or may be bound in other areas of the cell. To date, there is no report available for the mechanism of 5-FU penetration through cell membranes. Our experimental results implied that 5-FU rapidly accumulates in algae cells and potentially affects the aquatic biocenosis especially of primary producers. This study demonstrated that 50.2% of labelled 5-FU accumulates in algae cells and 27.3% remain free in the medium.

However, residual 22.5% 5-FU was lost during experimentation (degradation by organism) and analysis.

4.4.3 Distribution of 5-fluorouracil to algae DNA and RNA

The results of this study demonstrated the integration of 5-FU in both DNA and RNA of algae at 24 hours exposure. At the beginning of the 5-FU exposure, cell concentration was maintained approximately at 22×10^4cells/mL; which is 22 times concentrated than the previous experiments. This higher concentration of 5-FU was taken with the assumption that higher cell numbers could incorporate more 5-FU molecules although it is in contrast to OECD, 201 guidelines. Therefore, a correlation was found between an increasing number of cells and the higher incorporation of 5-FU and as a consequence a larger amount of genetic materials was obtained. As result a higher level radioactivity was found. After 24 hours incubation, cell concentration reached 115×10^4cells/mL wherein comprised 82% new cells. Therefore, DNA of 18% of the cells (parent cells) could not have incorporated 5-FU. But RNA of all cells in the population would have incorporated 5-FU because transcription is a normal cellular process of all living organisms (both parent and offspring) and could frequently incorporate 5-FU. This general hypothesis was revealed with higher accumulation of 5-FU in RNA than DNA. Approximately 0.41% and 0.59% of 5-FU of initial use was integrated into DNA and RNA respectively for the same quantity of cells (Table 6 and 7). As described in the materials and method section, 261.7 DPM radioactivity was measured in DNA of 30 mL cell culture. But most of the 5-FU molecules were un-accumulated and retained in the medium which showed 53730 DPM activities. Also a large amount of incorporated 5-FU passed through DNeasy mini elute column during the DNA extraction. The radioactivity captured in the waste fraction and the DNeasy mini columns were found to be 8385.7 DPM and 949.5 DPM respectively (Table 4-6). Thus, the total radioactivity obtained from the three sub samples was 9596.9 DPM. As for DNA extraction, the three sub-samples of total RNA extraction from 30 mL cell culture likely RNA, extraction waste and RNeasy mini column showed radio-activities of 363.2 DPM, 5253.9 DPM and 2689 DPM respectively (Table 4-7). The sum of these radioactivities was found to be 8315 DPM.

Table 4-6. Quntitative distribution (mean ± SD) of labelled 5- fluorouracil in cellular DNA and other sub-samples during DNA extraction, where n=3

Sample name	Radioactivity (DPM) in 30 mL culture	Radioactivity in 100 mL culture	% of distribution	Recovery
Control	0	0	0	210729
DNA	261.7 ± 39.8	872.4	0.41	
Extraction waste	8385.7 ± 194	27593	13.2	
Extraction Column	949.5 ± 126	3165	1.50	
Cell culture supernatant	53730 ± 445	179100	84.9	

Table 4-7. Quantitative distribution (mean ± SD) of labelled 5- fluorouracil in total RNA and other sub-samples during RNA extraction, where n=3

Sample name	Radioactivity (DPM) in 30 mL culture	Radioactivity in 100 mL culture	% of distribution	Recovery
Control	0	0	0	206818
Total RNA + 5-FU	363.2 ± 20.9	1211	0.59	
Extraction waste	5253.9 ± 34.1	17513	8.47	
Extraction column	2698 ± 202	8994	4.35	
Cell culture supernatant	53730 ± 445	179100	86.6	

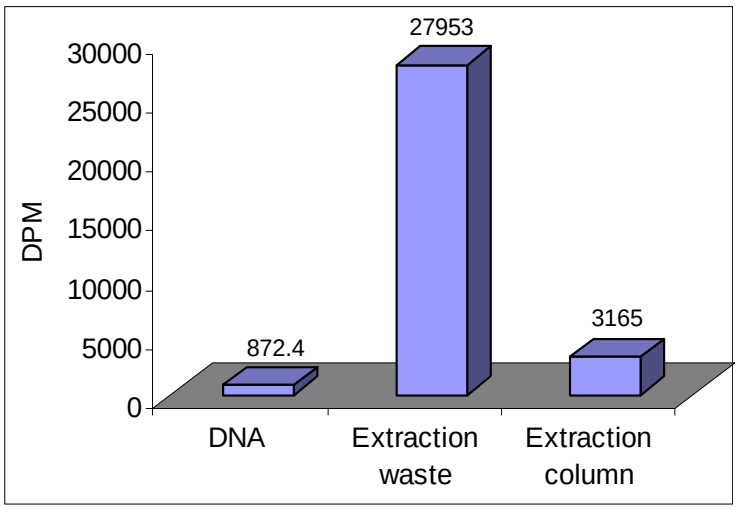


Figure 4-20. Accumulation of radio-labelled 5-FU in microalgae total DNA of 100 mL culture. Large percentage of 5-FU passed the column during DNA extraction, n=3.

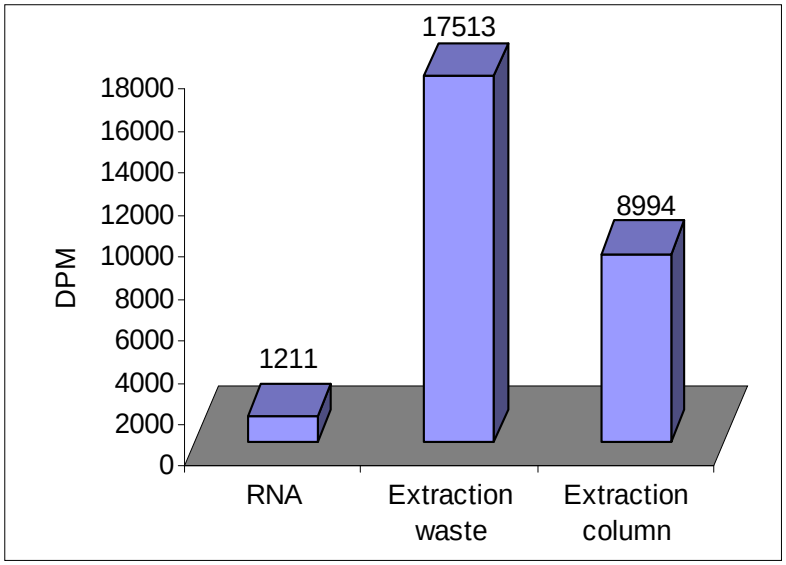


Figure 4-21. Accumulation of radio-labelled 5-FU in microalgae total RNA of 100 mL culture. Large percentage of 5-FU passed the column during RNA extraction although RNA could retain more 5-FU than DNA, n=3.

Some extent of radioactivity was typically found in the extraction waste and clean-up columns during the extraction of DNA and RNA. These amounts of 5-FU were considered as unspecific accumulation in the cells. In case of DNA extraction; the radioactivity of the three sub-samples (DNA, 872.4 DPM; extraction waste, 27953 DPM and column, 3165 DPM) of 100 mL culture contributed to 31990 DPM (or 14.41 nCi) which equals 35.5ng of 5-FU (Table 4-6 and Fig. 4-20). These measured radioactivities were attributed to the accumulation in algae cells. On the other hand, the sum of the radioactivity of the three sub-samples of RNA extraction was found to be 27718 DPM (or 12.49 nCi) which equals 30.7 ng of 5-FU (Table 4-7 and Fig. 4-21) which is also assigned to accumulation in cells. The comparison of radioactivity in extraction waste and purification column between DNA and RNA extraction process showed little variation. Those differences of activity may be caused by the performance and capacity of the DNeasy and RNeasy mini columns. However, in the case of the extraction of DNA and RNA about 14% labelled 5-FU was incorporated into the genome of algae or any other locations of the cells with slight variation. Thus, 86% labelled 5-FU was not incorporated and retained in the medium. The measurements of radioactivity for both DNA and total RNA from the same volume of cells were characterized by higher accumulation ratio of 5-FU in RNA compared to DNA. Cell systems (uracil-DNA glycosylase) rapidly remove fluorinated pyrimidine from the genome and continuously incorporate it into RNA which could be the possible reason of lower radioactivity in DNA compared to RNA.

5-FU is a rapidly uptaken by algae cells and then after integrated into genomic materials. 5-FU is incorporated into the genetic materials (Heidleberger, 1975, Donald *et al*., 1983) and causes the heterogenesity of ribosomal RNA formation as well as production of improper proteins in yeast (Mayo et al. 1968). Several mechanisms suggest that 5-FU is involved in the activation of ribonucleosides, namely 5-fluorouridine and its subsequent phosphorylation and incorporation into RNA (Suzanne and Morris, 1993). The exposure of radioactive 5-FU in algae culture demonstrated the incorporation into both DNA and RNA and also revealed the quantitative distribution of 5-FU between them.

4.4.4 Genetic transfer of labelled 5-FU into protozoan via algae genome

This experiment reflected genetic bio-transfer of labelled 5-FU and was identified in protozoan cells, *Tetrahymena pyriformis* via algae genomic material. The algal genomic material containing 469 DPM activity from incorporation of 5-FU corresponding to 24×10^{11} molecules of 5-FU (or 0.52 ng) fed to *Tetrahymena*. These cells were cultivated for 24 h and 48 h. In parallel; a control experiment was conducted for PPY-medium without cells. Cells continuously divide and reproduce in PPY-medium in addition with ingestion of radioactive algal genomic material. The cell growth was monitored by counting with a Neubauer cell counter and reached 1.6×10^{5}cells/mL and 1.4×10^{6}cells/mL after 24 h and 48 h incubation, respectively. This reveals almost 10 fold increase of cells over 24 h intervals. The ingestion of radioactive algal genomic material by *Tetrahymena* was ascertained with the exhibition of radioactivity in *Tetrahymena* cell pellets. Cell pellets of 5 mL culture expressed 45.1 DPM after 24 h incubation. Therefore, the entire culture evidenced 361 DPM. However, somewhat less radioactivity of 352 DPM was found after 48 h incubation in the entire culture (Table 4-8 and Fig. 4-22). On the other hand, much lower radioactivity was observed in PPY-medium without *Tetrahymena.* It was found to be 54 DPM and 35.5 DPM after 24 h and 48 h exposure, respectively.

Table 4-8. Transfer of labelled 5- fluorouracil which was integrated in algal genome to *Tetrahymena* after 24 hours and 48 hours exposure. Results were calculated as mean ± SD, where n=3

Name of sample	Extraction volume (mL)	Radioactivity (DPM)		Radioactivity in 40 mL culture		% of recovery	
		24 h	48 h	24 h	48 h	24 h	48 h
Control	5	0	0	0	0	0	0
T +5-FU	5	45.1 ± 7.1	44 ± 2.8	361	352	77	75
M+5-FU	5	6.7 ± 2.1	4.43 ± 1.1	54	35.5	12	7.6

Note: T and M means *Tetrahymena pyriformis* and PPY-medium respectively.

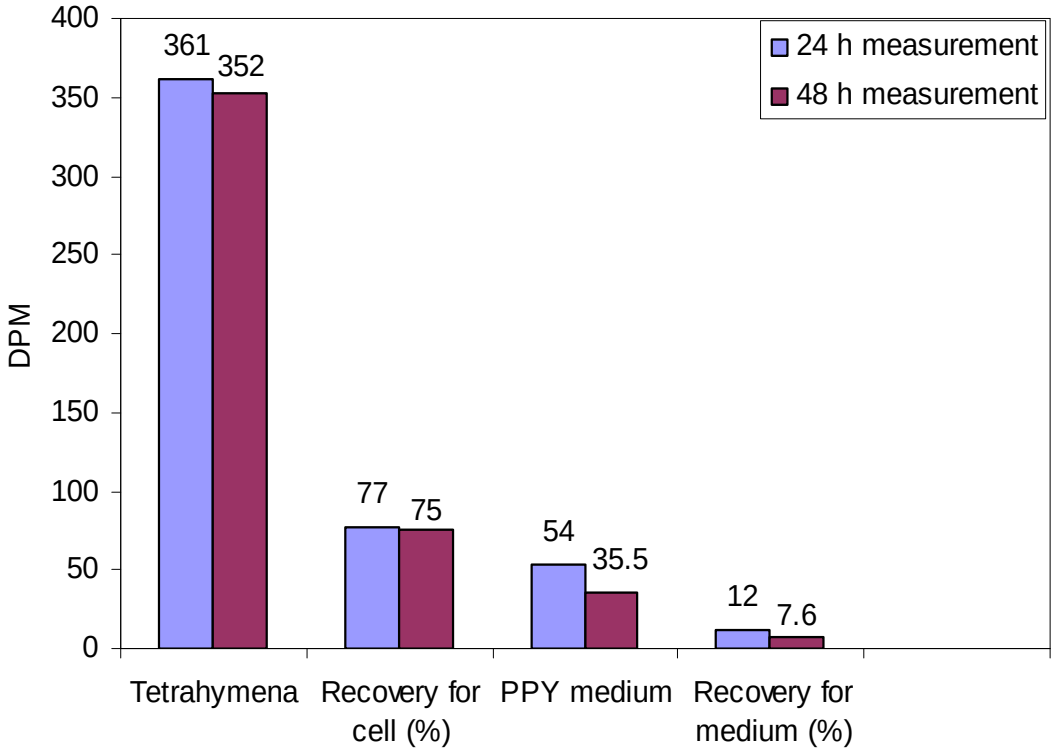


Figure 4-22. Transfer of radio-labelled 5-FU into protozoan, *Tetrahymena pyriformis* via genetic materials of microalgae in 40 mL culture. 77% 5-FU enters the *Tetrahymena* cell after 24 h exposures. But the existence of 5-FU was reduced 2% after 48 h exposure. Whereas only 12% and 7.6 % 5-FU was found in PPY-medium over 24 h and 48 h incubation respectively at same experimental conditions for the cells. Therefore, the large difference of 5-FU activity between cells and medium truly suggests that *Tetrahymena* cells ingested suspended genetic materials in the culture medium, n=3.

So, the cell pellet of the entire culture exhibited 361 DPM activity obtained from algal nucleic acid after 24 h incubation which reflected 77% recovery of initial applied radioactive material (Fig. 4-22). These genetic materials may form food vacuoles and remain stored for some time until exocytose. But, PPY-medium showed 54 DPM radioactivities corresponding to 12% of initial application after 24 h exposure. Therefore, the large difference of radioactivity between *Tetrahymena* cells and PPY medium absolutely indicates that *Tetrahymena* cells ingest radioactive genetic materials from the culture medium. The same experiments were conducted for 48 h incubation where the differences in radioactivity between cells and PPY medium were also prevalent. *Tetrahymena* cells exponentially grow and engulf higher amounts of radiolabelled genome. 24 h exposure of *Tetrahymena* revealed 77% radioactivity of initial use. Somewhat depleted (75 %) radioactivity was observed after 48 exposures, although the cell numbers increased about 10 times between 24 h and 48 h incubation intervals. This slight depletion of radioactivity may be caused by the degradation of ingested genome materials accompanied by decreasing concentration in the medium due to rapid uptake. The result

attributed that the *Tetrahymena* cell degrades ingested genome to a certain extend and it seems that the cell did not remain to fix the radioactive genomic material. Probably cells metabolized the ingested radioactive algae genetic materials and consequently excreted it into the culture medium. Therefore, our observations suggest that 5-FU can naturally bio-transferred from one aquatic species to other aquatic species which might appear as a hazard in the microcosm as well as in the food chain.

4.4.5 Reintegration of labelled 5-FU into protozoan DNA

The reintegration of radio-labelled 5-FU (bound in the algae genome) into the genetic materials of *Tetrahymena* were conducted similar to genetic transfer experiments. But in this experiment, DNA extracts were attributed to measurement of radioactivity instead of whole *Tetrahymena* cells. There was no significant activity found, although noise activity was detected. The noisy activity was assumed to be background and was not considered exactly for reintegration of 5-FU into genome of predator organisms.

The lacking radioactivity in the DNA of *Tetrahymena* could be explained by two points; first, labelled 5-FU was never reintegrated into the DNA of *Tetrahymena* under the experimental conditions chosen, and second, the protocol to determine radioactivity was not sensitive enough. However, if we consider the background radioactivity of in DNA of *Tetrahymena* then few radioactivity could be found in the DNA of *Tetrahymena* which corresponds to approximately 51×10^{10} molecule 5-FU. In addition, the fate of the algae genome ingested by *Tetrahymena* is a vital question. The ingested foreign genetic materials by the predator organisms could enable the formation of food vacuoles in the cell cavity. However, the foreign genetic materials accumulated in food vacuoles may be degraded into smaller fragments or oligonucleotides or even single nucleotides by the action of host enzymatic systems. For this reason, fragmented DNA has probably not been recovered during DNA extraction of *Tetrahymena*. The fragmented algae genome was passed through the DNeasy tissue column. The extraction column could not retain less than 100 bp DNA. Therefore it can recover only DNA of *Tetrahymena* without any digested foreign genome and shows no activity. The addition of several thousand DPM of algae genetic material into the *Tetrahymena* culture might cause some reintegration into the genome of *Tetrahymena*.

4.5 Toxicity assessment of PFCs to *Tetrahymena*

4.5.1 Toxicity effect of PFOA and PFOS

PFOS and PFOA caused an effect on *Tetrahymena pyriformis* growth. The selected concentration series (0 to 100 mg/L) represents high possible loadings of surface waters, which are habitats for many zooplanktonic species including protozoan. Studies focused on the concentration of PFCs in the environment referred about background concentrations ranging from 0 to 10 ng/L, but in case of some ecological accidents the concentrations could reach tens of mg/L. Similar studies made on human population stated similar findings in blood of normal population and workers from some factories, which produce PFCs. One literature claimed that vertebrates show stronger effects of PFOS in comparison with PFOA (Olsen *et al*., 1998). In this study, a similar response was recorded. Relatively high maximal concentration of PFOA (approx. 100 mg/L) was not sufficient to inhibit more than 40% of the growth rate in comparison to control sample (Fig. 4-23). To the contrary of this, PFOS caused at the same concentration nearly 90% or more inhibition of growth (Fig. 4-24). The effect of low concentrations was not significant, data showed either only very weak inhibition or even stimulation (0 ± 5%). The least significant differences between samples and control were recorded in concentrations higher than 25 mg/L for both compounds (ANOVA, Fisher-LSD test, P=0.05; data not shown).

Effective concentrations of PFOA and PFOS at different levels were established after 24 and 48 hours of exposure (Table 4-9). The length of exposure periods were chosen with respect to natural time border between exponential phase of growth and subsequent phase leading to the slower growth and a flashpoint of the culture.

Table 9. Effective concentrations of PFOA and PFOS evaluated from the growth response of *Tetrahymena pyriformis* after 24 and 48 hours of cultivation respectively.

Compound	Endpoint	Incubation time	EC values (mg L^{-1})
PFOA	EC_{20}	24 h	77.6 ± 15.3
		48 h	64.5 ± 13
PFOS	EC_{50}	24 h	93 ± 17.6
		48 h	72 ± 9.6

To improve their solubility, both compounds were dissolved in methanol/water system. Even though methanol at a concentration of 2% (vol.) exhibits toxic effect on *Tetrahymena*, the extent of inhibition did not exceed 20% (Fig. 4-25). Concentration series of both compounds were prepared either by direct dilution of their stock solutions in PPY medium (decreasing

concentration of methanol) or by pre-dilution once again in methanol/water solution and subsequent delivery to PPY (equal concentration of methanol).

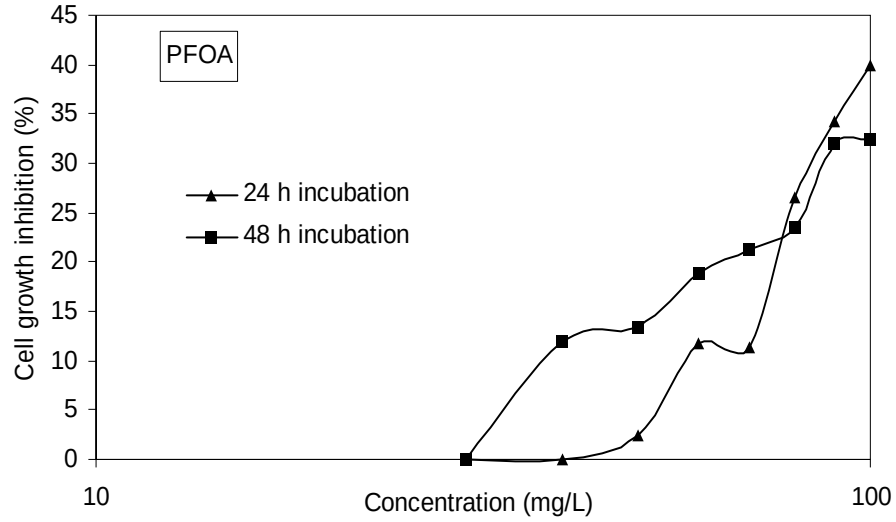


Figure 4-23. Mean inhibition of *Tetrahymena pyriformis* growth in the presence of PFOA after 24 hours and 48 hours of exposure. PFOA concentrations (0 to 100 mg L^{-1}) are expressed by means of a logarithmic scale. (n=6)

Samples exposed to decreasing methanol concentrations did not show any significant difference in comparison with those exposed to equal methanol treatment. Some authors (Sanderson *et al.*, 2004) describe effects of toxic compounds on *Tetrahymena* morphology, but in case of PFOA and PFOS we did not record any significant change, because even the cells in the control sample were of different size and shaped from standard oblong *Tetrahymena* cell to more or less rounded cells.

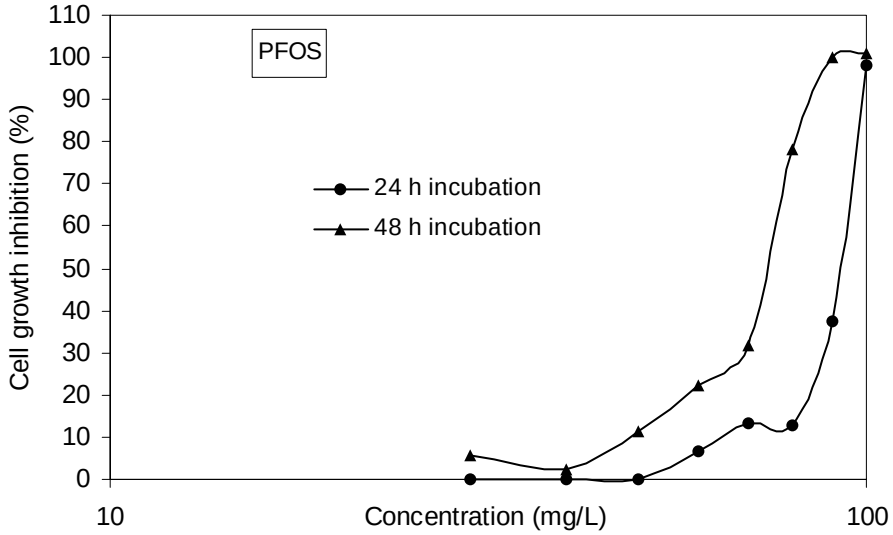


Figure 4-24. Mean inhibition of *Tetrahymena pyriformis* growth in the presence of PFOS after 24 hours and 48 hours of exposure. PFOS concentrations (0 to 100 mg L^{-1}) are expressed by means of a logarithmic scale. (n=6)

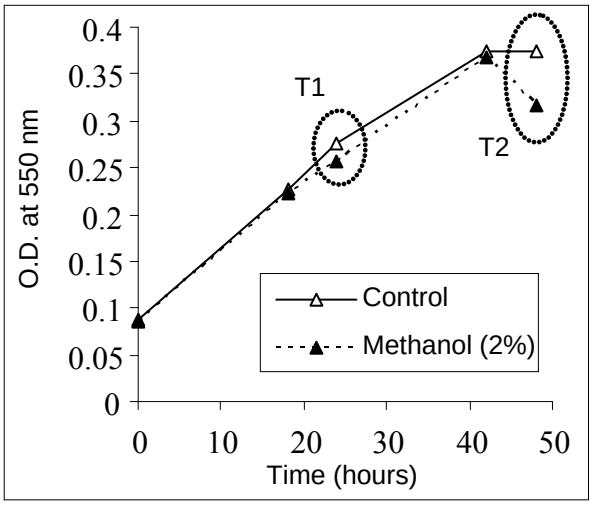


Figure 4-25. Comparison of growth curve of control and methanol (2% vol.) treated sample in the course of 48 hour exposure period. Symbols T1 (24 h), resp. T2 (48 h) mark the time points of PFCs toxicity measurements.

One of the factors influencing PFCs availability for organisms could be the state of surfactant molecules in the solution. More hydrophobic species of perfluorinated surfactants, like PFOS and its tetraethylammonium salt (TEA-PFOS), are known for their ability to create micelles in aqueous solutions. The formation of micelles is dependent mainly on the concentration of the surfactant. By means of various methods it is possible to find a so-called critic micelle concentration (CMC) which is typical for each compound and some physico-chemical conditions of the environment – the temperature, charge and other factors such as the presence of various ions e.g. from dissolved salts. López-Fontán *et al*., 2006 and 2006; Dias *et al*., 2003 observed that CMC value for TEA-PFOS at 28°C is approx. 1mM. Nevertheless the starting point of micelle formation could be lower, around 477 μM, when the first ion pairs of $PFOS^-$ and TEA^+ occur. A study with another protozoan *Paramecium caudatum* (Matsubara *et al*., 2006) showed that a time period of stress-induced backward swimming of this protozoan is in good correlation with CMC value of the tested compound. Compounds with low CMC could be less toxic than those with high value of CMC like PFOS.

4.5.2 Membrane damage of *Tetrahymena* to PFOA and PFOS

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant when the plasma membrane is damaged. An increase in the amount of dead or plasma membrane-damaged cells results in the increase of LDH activity in the supernatant culture (Goergen *et al*., 1993; Legrand *et al*., 1992). This increase in the amount of enzyme activity in the supernatant directly correlates to the leakage

of membrane during a limited time period. Therefore, the amount of color released in the assay is proportional to the number of lysed cells. There are many environmental toxic compounds that have been studied regarding to membrane damage of cells. The organic compounds are known to damage cell membranes, alter the function of integral membrane proteins (Tahti, 1992; Sikkema *et al*., 1994; Engelke and Tahti, 1996; Urban, 2004), with a correlation between toxicity of particular compounds and their hydrophobicity (Dreiem *et al*., 2003). However, PFCs are also known as moderate membrane permeable compounds due to the fatty acid analogs. As a result, they diffuse into the lipid bilayer and thereby cause membrane fluidity (Hu *et al*., 2003; Starkov and Wallace, 2002). Here we describe for the first time the membrane damage of *Tetrahymena pyriformis* as indicated by LDH leakage resulting from the exposure to PFCs in a concentration-dependent manner.

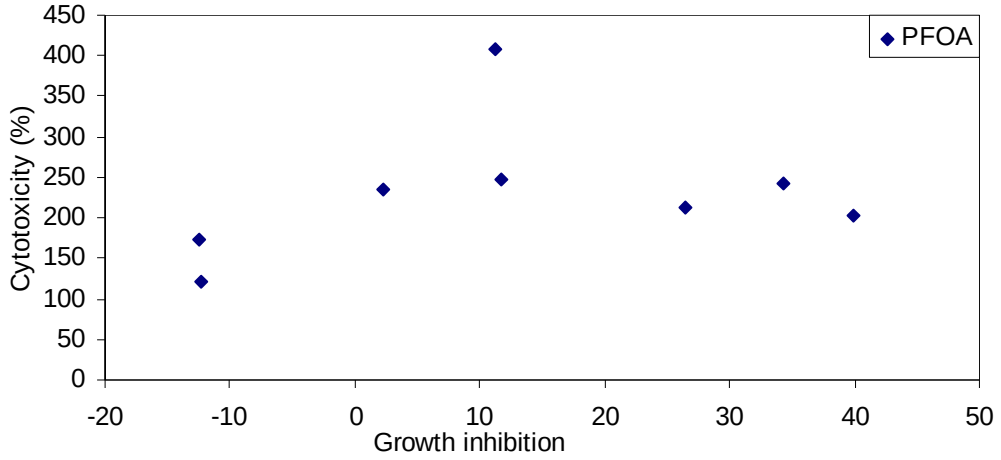


Figure 4-26: Correlation between cytotoxicity (membrane damage) and growth inhibition of PFOA after 24 hour incubation. A higher increase of membrane damage was found with moderate cell growth inhibition.

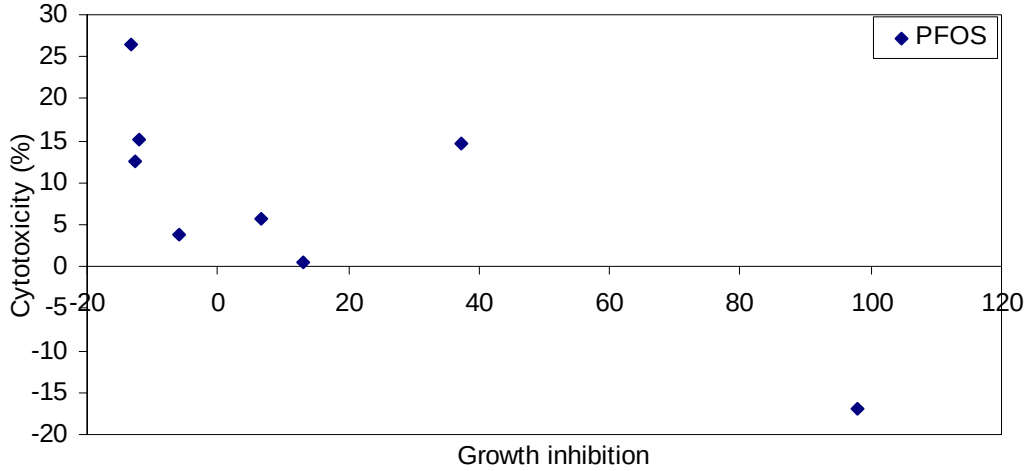


Figure 4-27: Correlation between cytotoxicity and growth inhibition of PFOS after 24 hour incubation. A lower cytotoxicity was found with comparatively higher cell growth inhibition. The exposure of PFOA and PFOS to the *Tetrahymena* cell induces membrane damage

following acute exposure. This causes LDH leakage from the cell cytoplasm which was detected by the cytotoxicity kit. No significant change in LDH leakage was observed in 2% methanol control cells (P>0.05), data not shown. However, the LDH leakage response of PFOA was greater than that of PFOS at the same concentration range which is counterpart to the cell growth inhibition (Fig. 4-26 and 4-27). This study revealed that higher concentrations of PFOA induce more damage of the membrane and as consequence release LDH into the culture medium. Though, PFOA expressed less cell growth inhibition as compared to PFOS. On the other hand, PFOS exerted higher cell growth inhibition with exhibition of unexpected low LDH leakage. Interestingly (Liu *et al*., 2007) demonstrated that a dose-dependent increase in the lipid per-oxidation (LPO) level was observed only in the PFOA exposure groups, whereas LPO remained unchanged in the PFOS exposure groups. This finding could be a possible explanation of opposite cytotoxicity of PFOA and PFOS in correlation with cell growth inhibition. However, the most likely mechanism to explain the moderate membrane damage of the moderately lipid-soluble PFCs is that they have lower dipole moments compared with the non-perfluorinated hydrocarbons. Therefore, PFCs such as PFOA and PFOS with their low solubility in aqueous phases (log P ~7), are more likely to partition in the center of the bilayer compartment of the membrane. As such, they exert a minimal effect on lipid fluidity and ordering within the membrane bilayer (Smith *et al*., 1997; Smith *et al*., 1995; Steinhorn *et al*., 1995; Woods *et al*., 2000). In addition, reactive oxygen species (ROS) formation was found to be responsible for the increased LDH leakage noted in cells exposed to organic compounds (Dreiem *et al*., 2005) and may explain the increase in LDH leakage. As similar PFOA is able to produce oxidative stress and induce apoptosis with the generation of ROS (Panaretakis *et al*., 2001).

4.5.3 Toxicity of FTOH to *Tetrahymena thermophila*

Four perfluorinated alcohols such as 4:2 FTOH, 6:2 FTOH, 8:2 FTOH and 10:2 FTOH were tested in *Tetrahymena thermophila*. However, all the FTOH could not significantly inhibit the cell growth for 24 h and 48 h exposure. Probably, the perfluorinated alcohols do not affect the membrane of the cell. The tests were repeated many times for all the compounds. Sometimes 6:2 FTOH, 8:2 FTOH exhibited EC_{10} values but that results were very unpredictable. On the other hand the test of 4:2 FTOH was difficult due to is volatility. Therefore, perfluorinated alcohol could be considered as nontoxic or very little toxic to the *Tetrahymena* cell.

4.5.4 Damage of macronucleus of *Tetrahymena thermophila* by FTOH

Tetrahymena thermophila cell was stained with acridine orange after exposure to perfluorinated alcohols. This dye binds to DNA in the macronucleus which causes green fluorescence at 488 nm ($\lambda_{emission}$). All the subsamples of cells were incubated for 48 h. The morphology of the control cell was normal, and the macronucleus seen as complete shapeand size, showing green fluorescence and located at the middle of the cell. Macronucleus was indicated by an arrow (Fig. 4-28). The negative control (methanol) did not damage the macronucleus thus exhibiting similar result as for normal control cells. On the other hand, phenol was administered as positive control which causes the damage of macronucleus in the cell. Surprisingly, the exposure of PFCs caused the identical damage of the macronucleus as phenol. After the exposure of PFCs some cells showed the larger macronucleus or conceal the macronucleus as depicted in the Fig. 4-28. FTOH may induce chemical signals in the cells which cause the dysfunction of DNA and consequently influence shape of macronucleus in the cell.

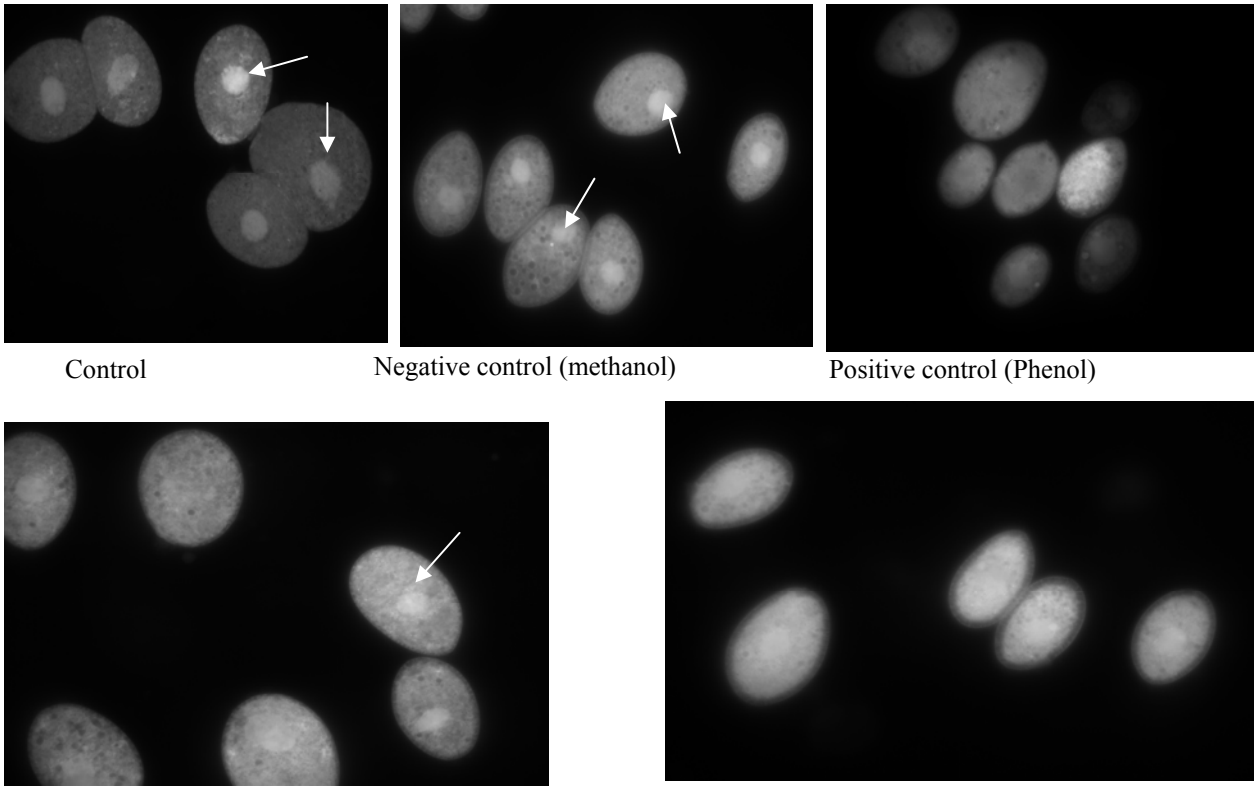


Figure 4-28: Fluorescence image of *Tetrahymena thermophila*. The macronucleus appeared as green (indicated by arrow) due to the staining with acridine orange.

4.6 Effect of nanoparticle TiO_2 to *Tetrahymena thermophila*

The growth effect of TiO_2 to *Tetrahymena* population was assessed by means of counting of their number by Neubauer counting chamber after 0, 5, 24, 29 and 28 hours. This growth inhibition test was performed with *T. thermophila* and TiO_2 nanoparticles lower than 10 nm in size. That TiO_2 exhibits only low toxic effect even in very high concentrations of the nanoparticles applied in the cultivation medium (up to 400 mg/L). The cell growth effect of this nanoparticle could not be evaluated due to the insignificant result. However, this study did not thoroughly evaluate the toxicity of nanoparticle using nominal concentration due to the insolubility. Nanoparticles only give a suspension in water by ultra-sonication (by definition particles give no solution) which undergo sedimentation after a certain time if the suspension is not stabilized by intermolecular interactions with the solvent or adjuvant chemicals. Interestingly, the microscopic studies of TiO_2 provided an exciting information about its fate inside protozoan cells. The protozoan cells are able to the ingestion of TiO_2 particles, but can not digest them. Microscopic study revealed that *Tetrahymena* immediately ingest the TiO_2 particles and form food vacuoles wherein they store them until exocytose into the medium as depicted in the Fig. 4-29. In the food vacuoles the TiO_2 undergoes agglumeration and is subsequently released as a bigger aggregate. The particles form aggregates randomly with *Tetrahymena* growth which leads to larger particle aggregates over time. On the other hand, the nanoparticles can also get self aggregated by influencing environmental factors producing small aggregations as compared to cells. Therefore, *Tetrahymena* can act as a factor to influence the bio-aggregation of nanoparticles. These results may provide a message about the fate of nanoparticles in the aquatic environment.

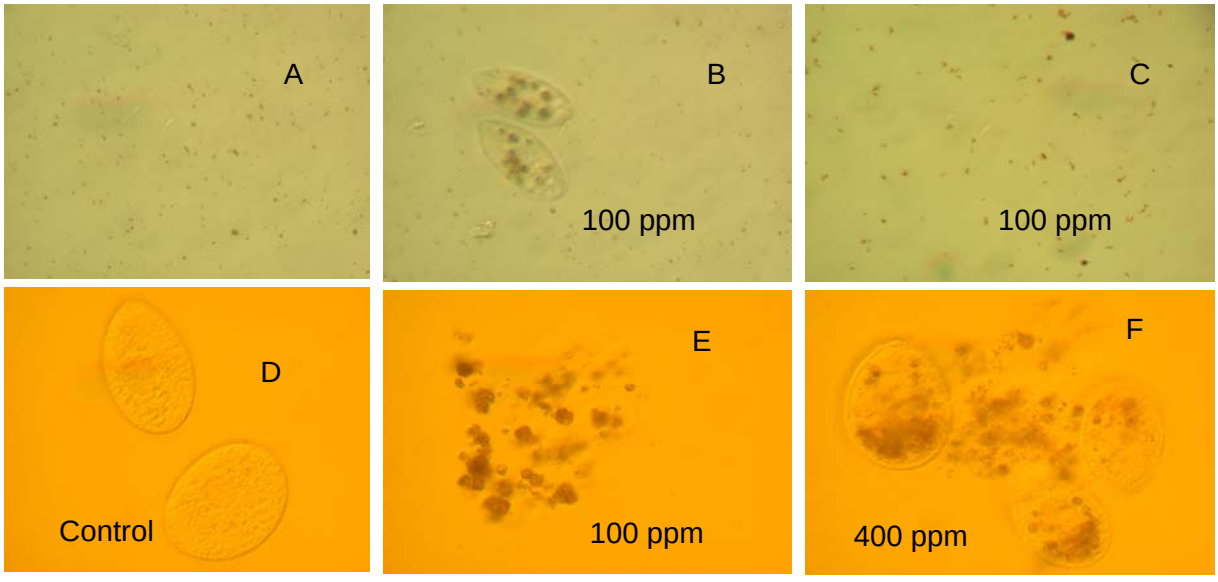


Figure 4-29: Effect of nano TiO_2 to *Tetrahymena thermophila*. Cell ingests free small NPs and excretes them as bigger aggregates. The images A and B indicate the suspension of NP in PPY medium and cell culture respectively after 30 min. however, the image reveal that the cells immediate incorporate the NPs. Image C indicates the natural aggregation of NP in the PPY medium after 48 hour exposure. The image D indicates the control cells after 48 hours. Interestingly, images E and F show the effect on NP by cells after 48 hours incubation. This clearly demonstrates that *Tetrahymena* cells widely influence the agglomeration of NP and release into the medium.

However, many NMs and the scientific community is making observations on NPs ecotoxicity to inform the wider debate about the risks and benefits of these materials. There are concerns that natural nano-scale process could be influenced by the presence of pollution (Handy *et al*., 2008). NPs comprise two risks, there must be both a potential for exposure and a hazard that results after exposure. In the environment the formation of aggregates of NPs and therefore of larger particles that are trapped via many factors (Nowack and Bucheli, 2007). Abiotic factors such as pH, ionic strength, water hardness and the presence of organic matter will alter aggregation chemistry; and are expected to influence toxicity (Handy *et al*., 2008). The physico-chemistry is essential to understanding of the fate and behaviour of NPs in the environment, as well as uptake and distribution within organisms, and the interactions of NPs with other pollutants. Aggregated or adsorbed NP will be less mobile, but uptake by sediment-dwelling animals or filter feeders is still possible. This study reveals that *Tetrahymena* spec. spontaneously engulf the nanoparticles, and magnify the degree of aggregation. The effect of different form of nanoparticles in the *Tetrahymena* is depicted in the fig. 4-30.

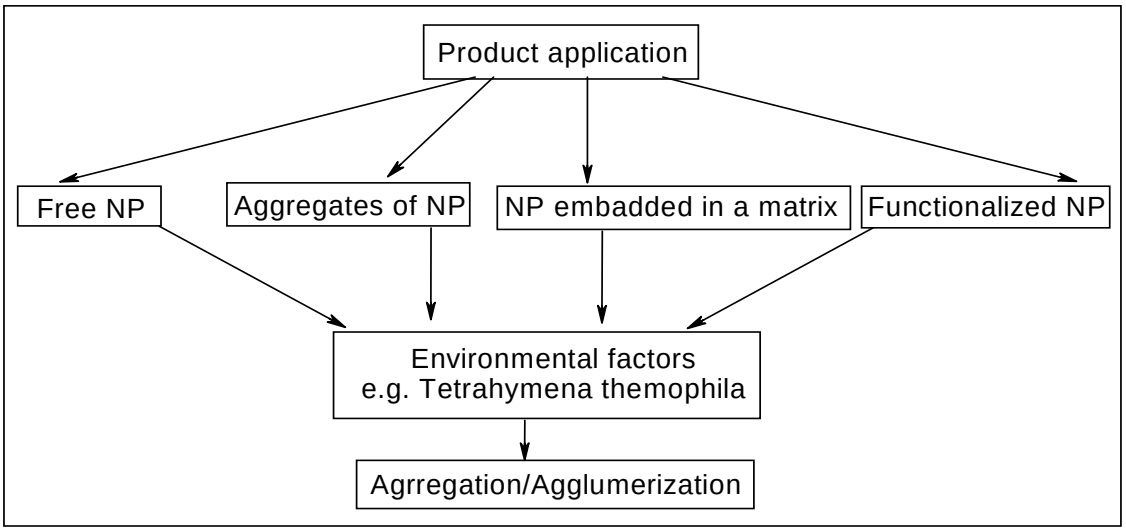


Figure 4-30: *T. thermophila* fed all form of NP released from products and liberates as aggregates.

5. Conclusion of research

This investigation established a high throughput test for catecholamines and other compounds and evaluated their toxicity and metabolism in the ciliate protozoan, *Tetrahymena pyriformis*. All tested catecholamines represented moderate acute toxicity in *Tetrahymena* and depleted the number of cells. Due to the production of ROS, catecholamine interferes to chemical signaling inside the cell. Their presence seems to imbalance the normal production of catecholamines. The mechanism of this toxicity is probably a consequence of auto-oxidation and the generation of toxic quinones. The concentration of all representative catecholamines was measured by HPLC-ED as sensitive analytic tool. Noradrenaline was produced to higher concentrations as compared to other monoamines in the natural *Tetrahymena* culture in 96-well plate system. However, the production of all biogenic monoamines reached maxima during the exponential growth of cells possibly due to the higher activity of cellular enzymes. Therefore, biogenic monoamines especially noradrenalin might be considered a useful bio-indicator for cell proliferation and other cellular activities. On the other hand biogenic monoamines were observed to be highly degradable in the 96-well plates. Parallel exogenous exposure of catecholamines at low concentration to the cells surprisingly depleted noradrenaline concentration. These effects may be accompanied by reluctance in the cell system and no longer synthesis dopamine and subsequent catecholamines. This effect would suggest the substrate inhibition of dopamine β hydroxylase which is responsible for noradrenaline synthesis. Nevertheless, all tested compounds could affect the noradrenaline synthesis and causes the decreased growth/reproduction rate of cells as well as presented acute toxicity in the cells. The toxicity may be caused by enzyme inhibition of dopamine β hydroxylase that may propel cells to the generation of the metabolite. Therefore, dopamine causes higher activity of enzyme inhibition which may enforce the cell system to the generation of a novel unidentified metabolite. This metabolite formation is biotic and enzyme dependent.

The metabolite of dopamine was characterized employing UPLC-MS and FTICR-MS. Both analytical tools were developed as highly sensitive methods for analysis of dopamine and catecholamine in *Tetrahymena* extract by optimizing separation and determination conditions. The chosen LC-MS set up appears to be a good compromise between the demand for highest sensitivity and accuracy, as well as lowest investment of time and running costs. This LC-MS uses positive electrospray ionization. However, the UPLC unambiguously separates the analytes in the cell extract as similar order of HPLC-ED where the novel metabolite always

eluted later. Consequently the separated analytes injected into the ESI-MS provide two peaks; the earlier peak demonstrated the dopamine $[M+H]^+$, 154 m/z and the later peak demonstrated the metabolite $[M+H]^+$, 151 m/z. On the other hand, the high resolution FTICR-MS enables an accurate mass determination of dopamine and its metabolism. The calibration of FT-MS data allowed further improvement of the analytical precision of the setup. The same positive electrospray ionization was applied and could recognize dopamine and metabolites similarly to UPLC-MS and consequently reveals closely related molecular formula. The proposed molecular formula of the metabolite is X and corresponds to the actual molecular weight of 150. However, here the proposed possible structure of the novel metabolite was assumed based on literature study and on our experimental results. In the oxidation environment, one hydroxyl group can be splitted from the benzene ring and a hydroxyl group still remains at the aromatic ring of dopamine and thereby formed tyramine (Coulon *et al*., 1994; Hiroi *et al*., 1998). The side chain (primary amine group) of tyramine can be again oxidized to produce imidate salt (Weintraub *et al*., 1968). The imidate salt could be converted into amidine compounds in ammonia solution. However, the complete structure elucidation was not possible due to the short time schedule of this thesis. Nevertheless, the hypothesis suggests the possible name and structure of the novel metabolite as depicted in the Fig. 5-1.

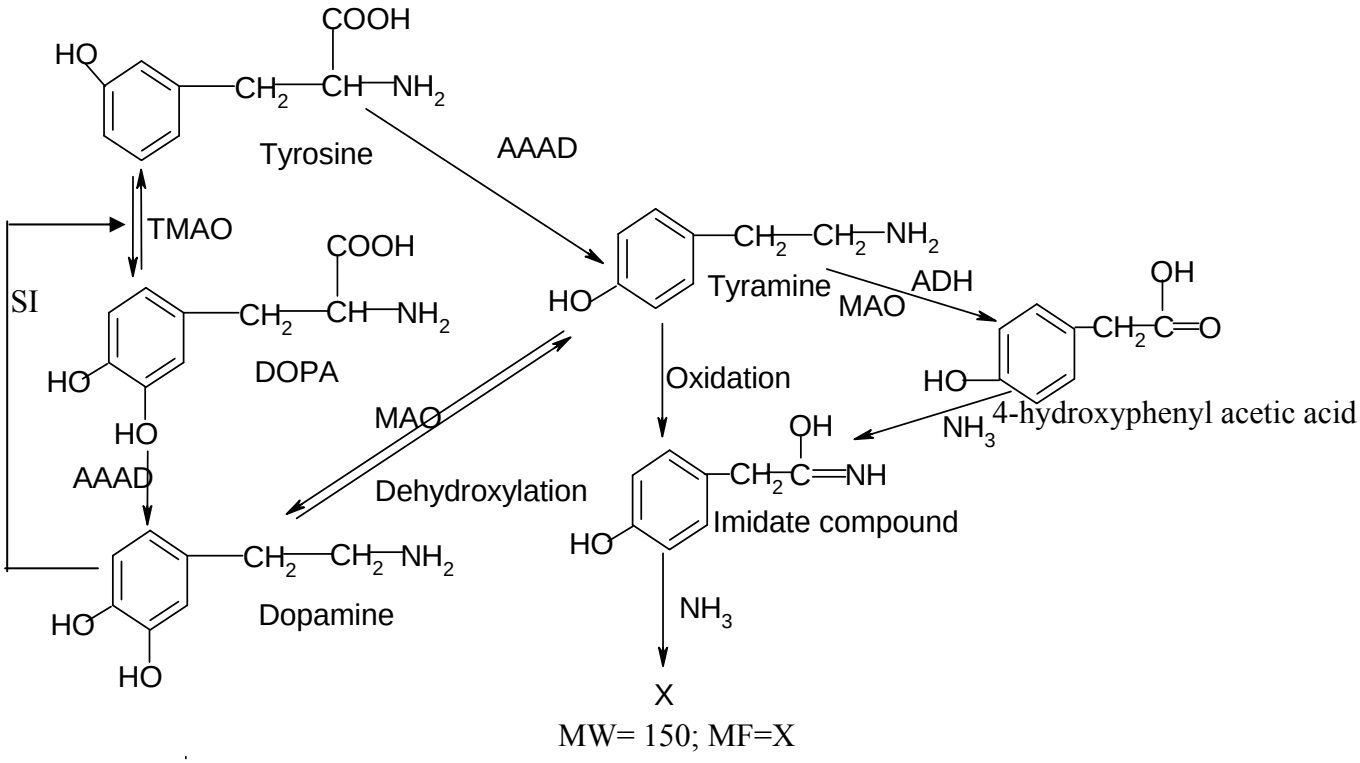


Figure 5-1: Proposed biosynthetic pathway and possible structure of the novel metabolite. Tyrosine-3- monoamine oxidase (TMAO); L-aromatic amino acid decarboxylase (AAAD); Monoamine oxidase (MAO); Aldehyde dehydrogenase (ADH); Substrate inhibition (SI).

The novel metabolite might play an important role in the field of neurodegenerative diseases such as Parkinson disease where L-DOPA is used in the treatment of patients (Standaert and Youg, 2006; Chen and Shimomura, 2000). It would be again reasonable to address the question whether there is the same metabolite generated in the treatment of L-DOPA and which role it is playing there. Our investigation also suggested that the *Tetrahymena* cell could be useful as a model for research into the basic neurotransmitter mechanisms at the cellular and molecular levels on the basis of our results and to further clarify the above mentioned hypothesis.

In recent years, a great deal of progress has been made on the characterization of dopamine receptors of invertebrates and those works have given rise to many interesting questions. The molecular, genetic, and behavioral tools available in invertebrates are particularly well suited to address such questions and promise, therefore, with great enhancement of understanding of the roles of dopamine. Interestingly, this investigation reported here for the first time the evidence of a dopamine receptor in the ciliate protozoan *Tetrahymena thermophila*. However, this investigation indicated only a D1 type receptor with the use of D1 specific fluorescent SKF-38393. However, dopaminergic cells contain D1 and D2 type receptors together. Thus, a D2 receptor might also be present in the *Tetrahymena* cell. Therefore, dopamine receptor genes are expressed in the exponentially growing *Tetrahymena* cell. In addition, the invertebrates *Caenorhabditis elegans, Drosophila melanogaster and Apis mellifera* strongly support a model where these two receptors can be co-expressed in the same cells, and suggest that the receptors act in opposition to each other by activating antagonistic signaling pathways. Thus, the single cell ciliates, *Tetrahymena thermophila* can also join this group of invertebrates as a model organism for dopamine receptor research as well as contributing to the understanding of other phylogentic type of neurotransmitters receptors mechanisms.

The study also evaluated the toxicity of 5-FU to microalgae and ciliated protozoan and indicates a risk of harm to the aquatic microbial community. 5-FU exhibited moderate acute toxicity in green microalgae and ciliated protozoan. 5-FU could elicit higher growth inhibition in protozoan than in algae. Small amounts of 5-fluorouracil could enter into the microalgae probably via the lipophilic interaction and accumulates in the cell. The bioaccumulation of 5-FU was revealed by the exposure of radio-labelled [2-^{14}C] 5-FU. Algae cells can rapidly accumulate 5-FU wherein a small percentage was integrated into the DNA and RNA of cells.

Tetrahymena fed radioactive genetic materials of algae through ingestion which was verified by the measurement of radioactivity in *Tetrahymena* cell pellets. No reintegration of labelled 5-FU was found in the DNA of predator organisms. However, we propose that the application of higher amount of labelled genome in the feeding organisms may lead to some measurable reintegration into the genome. Indeed, it would be also convenient to add cells whole algae to the predator organisms. Unfortunately, we could not carry out these experiments due to practical difficulties to separate *Tetrahymena* from algae afterwards. Nevertheless, this study demonstrated for the first time the adverse effect of 5-FU on microbial ecological part of networks and its bio-transfer from one organism to another organism in aquatic life as well as spreading throughout the ecosystem.

Perfluorinated compounds cause cell growth inhibition of *Tetrahymena*. However, effective concentrations of these compounds are recorded in orders of magnitude much higher than the usual environmental loading and occurrence. Growth inhibition tests with *Tetrahymena pyriformis* showed, that this protozoan is sensitive to PFOA and PFOS. Both these compounds exhibited slight acute toxic effects on protozoan growth, but the effect of perfluorooctane sulfonate was more significant than in case of PFOA. The perfluoro telomeric alcohols, FTOH did not exert significant cell growth inhibition at similar experimental conditions of PFOA and PFOS. In addition, PFOA and PFOS cause moderate membrane damage of *Tetrahymena* which was investigated by the measurement of LDH in the culture medium. The study also introduces the effect of nanoparticles into the aquatic environment. The use of TiO_2 to the culture of cell reveals that *Tetrahymena* endocytosed TiO_2 and form food vacuoles in the cell. This type of nanoparticle is stored in the food vacuole until exocytosis into the medium. However, the nanoparticle is released in large aggregates and the degree of aggregation is enhanced by *Tetrahymena* cell.

Therefore, in briefly, this thesis established the toxicity of catecholamines to *Tetrahymena*, and noradrenaline was synthesized in higher concentration. Exogenous exposure of dopamine and L-DOPA cases the liberation of a novel metabolite from *Tetrahymena*. The molecular weight and formulae of this new metabolite was determined as to150 m/z and its elemental formula to $C_8H_{10}N_2O$, respectively. In addition this study revealed the toxicity of 5-FU to *Tetrahymena* and microalgae *Scenedesmus*. Both of these organisms are sensitive to 5-FU. This compound could also be transferred from one organism to another organism via genetic material integrated 5-FU. The analysis of the ligands binding to the dopamine receptors

reveals the expression of receptor proteins. It was newly indentified the existence of one dopamine receptor in *Tetrahymena thermophila*. Further, it was investigated the effect of persistent perfluorinated compounds to *Tetrahymena*, concluding that these compounds can damage the cell membrane by interfering with the lipid bilayer and it was demonstrated the fate and effect of selected engineered nanoparticles in *Tetrahymena* species.

Future research options

Regarding to the development of high-throughput testing system for catecholamines and other compounds, the following points could be addressed in the future research.

- Further structural elucidation of the new dopamine metabolite.
- Assessment of the same metabolite in higher organisms.
- Determination of pharmacological properties of this metabolite with respect to dopamine in higher organisms.
- Investigation of dopamine receptor genes (D1 and D2 type) in *Tetrahymena thermophila* SB10 and sequencing of these genes.
- Evaluation of phylogenetic position of *T. thermophila* based on catecholamine and dopamine receptor gene.
- Investigation of homologues sequences for the gene of dihydropyrimidine dehydrogenase (DPD), determination of quantitative level of mRNA of this gene after exposure of 5-FU to *T. thermophila* and sequencing of CDS.
- Catecholamine homeostasis in *Tetrahymena thermophila* exposed with perfluorinated compounds and nanoparticles.

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