ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

OCCURRENCE, FATE AND EFFECTS OF PHARMACEUTICALS AND HORMONES IN AQUATIC ENVIRONMENT

Ph.D. THESIS

Egemen AYDIN

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SUCUL ORTAMLARDA İLAÇ VE HORMONLARIN SAPTANMASI, DAVRANIŞ VE ETKİLERİ

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Egemen AYDIN, **a Ph.D.** student of ITU **Graduate School of Science, Engineering and Technology,** student ID 501052703, successfully defended the **thesis** entitled "**OCCURRENCE, FATE AND EFFECTS OF PHARMACEUTICALS AND HORMONES IN AQUATIC ENVIRONMENT**", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my parents and sister,

FOREWORD

Everything started in 2003 when I stepped into the room of Prof. İlhan Talınlı as a just graduated, poor, and naïve boy. He shaped me up with his great wisdom and intellect like a perfectionist artist. I learned from him that how to reach to knowledge and how to convert knowledge to wisdom. I owe him and I will owe him all of my achievements in my academic life.

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April 2012 Egemen AYDIN

Environmental Engineer

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ABBREVATIONS

AGP	auxiliary gas pressure
APCI	Atmospheric Pressure Chemical Ionization
APPI	Atmospheric Pressure Photoionization
ATC	Anatomical Therapeutic Chemical
BOD	Biochemical Oxygen Demand
CE	Collision Energy
COD	Chemical Oxygen Demand
CP	Collision Pressure
CT	Capillary Temperature
DOT	Days of Treatment
E1	Estrone
E2	Estradiol
E3	Estriol
EC10	10% effective concentration
EC50	50% effective concentration
EC80	80% effective concentration
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EE2	17α-ethynylestradiol
ELISA	Enzyme-Linked Immunosorbent Assays
ELRA	Enzyme-Linked Receptor Assay
ESI	Electrospray Ionization
GC	Gas Chromatographer
GC-MS/MS	Gas Chromatography Coupled with Tandem Mass Spectrometer
HAA	Hormonally Active Agents
IDL	Instrument Detection Limit
ISGP	Ion Sweep Gas Pressure
LC	Liquid Chromatographer
LC-MS/MS	Liquid Chromatography Coupled with Tandem Mass Spectrometer
LOD	Limit of Detection
LOEC	Lowest Observable Effect Concentrations
LOQ	Limit of Quantification
MDL	Method Detection Limit
MQL	Method Quantification Limit
MS	Mass Spectrometer
MTBE	Methyl-tert butyl ether
NI	Negative Ionization
NOEC	No Observable Effect Concentrations

NRC	National Research Council
NSAID	Non-steroidal Anti-inflammatory Drugs
OECD	Organisation for Economic Co-operation and Development
PI	Positive Ionization
PNEC	Predicted No Effect Concentration
PPCPs	Pharmaceuticals and Personal Care Products
RIE	Relative Inductive Efficiency
RSD	Relative Standard Deviation
SGP	Sheath Gas Pressure
SPE	Solid Phase Extraction
SV	Spray Voltage
TLO	Tube Lens Offset
UPLC	Ultra-Performance Liquid Chromatographer
USEPA	United States Environmental Protection Agency
WHO	World Health Organization
YES	Yeast Estrogen Screen

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OCCURRENCE, FATE AND EFFECTS OF PHARMACEUTICALS AND HORMONES IN AQUATIC ENVIRONMENT

SUMMARY

The aim of this study is to provide specific information on the occurrence, fate and effects of pharmaceuticals and hormones in aquatic environment. 10 widely used pharmaceuticals (three NSAIDs, Diclofenac, Ibuprofen, Naproxen; four antibiotics amoxicillin, ciprofloxacin, erythromycin, and sulfamethoxazole; two β-blockers, atenolol and propranolol; and one stimulant, caffeine and 4 estrogen hormones estrone (E1), estradiol (E2), estriol (E3), and 17α-ethynylestradiol (EE2) were selected according to one year-sales data. The occurrence of selected pharmaceuticals and hormones in surface water in Istanbul, Turkey was investigated in this study. An important drinking water source, Büyükçekmece Lake and five main rivers flowing into the lake were selected for the monitoring of the compounds. Sampling was conducted five different times in a year in order to observe seasonal changes. A new, rapid and sensitive method using solid phase extraction and ultra-performance liquid chromatograph coupled with triple quadrupole tandem mass spectrometer was developed. Minimum quantification limits were between 0.5 and 1.1 ng/L for different compounds. Recoveries were between 72-119 % and 61-98 % for ultra-pure water and for surface water, respectively. All selected compounds were detected at least once in the samples. Some pharmaceuticals were detected as high as a few of micrograms per liter levels in the rivers. Most frequently detected compounds were caffeine and antibiotics (amoxicillin, ciprofloxacin, erythromycin and sulfamethoxazole). Synthetic hormone (17α-ethynylestradiol) was detected only 4 times making it the least detected compound in the whole sampling period.

Since pharmaceuticals are designed to exert biological effects, it is expected that they adversely affect ecosystem. Moreover, they may pose threat to human health via food web and/or direct exposure. Different tools were used for the determination of ecological impacts of selected pharmaceuticals and hormones to cover different effects and to understand responses of different species in different levels of the food web. *P. subcapitata* was used for the determination of acute effects whereas *D. magna* was used for the determination of both acute and chronic effects. Mutagenic effects and endocrine disruptive effects were determined with AMES and YES test, respectively.

The results of tests conducted with *P. subcapitata* and *D. magna* indicate that even though studied pharmaceuticals and hormones may not present acute adverse effects at low concentrations; they may have drastic chronic effects.

In addition to studying the effects of single compounds, the effects of mixtures of pharmaceutical and hormones were also studied since there was a lack of data in the scientific literature. All mixtures had synergistic interaction for *D. magna* acute immobilization, *P. subcapitata* growth inhibition, and *D. magna* reproduction inhibition tests. Moreover, mixtures had stronger toxicity than predicted values even

at concentrations at which single compounds do not exhibit effects for *D. magna* acute immobilization, *P. subcapitata* growth inhibition, and *D. magna* reproduction inhibition tests. These results indicate that NOECs for single toxicity tests are not enough for the assessment of environmental risks of the compounds since they will be present as a mixture.

SUCUL ORTAMLARDA İLAÇ VE HORMONLARIN SAPTANMASI, DAVRANIŞ VE ETKİLERİ

ÖZET

1960'ların ortalarından başlayarak PCBler, DDT ve metil civa gibi kirleticilerin zehirlilik etkilerinin ve besin zincirinde üst basamaklara çıkıldıkça canlılarda daha fazla biriktiğinin belirlenmesi ile kirleticilerin ekosistem üzerindeki zararlı etkileri ve çevredeki değişimleri konusundaki çalışmalar giderek artmaya başlamıştır. Bu tip tekil kimyasalların KOİ ve BOİ gibi kolektif organik parametrelerden farklı olarak tanımlanabilmesi için "özel su kirleticileri" (specific water pollutants) kavramı ortaya atılmıştır. OECD'nin yaptığı tanıma göre belirli koşullar altında suyun kalitesini özellikle insana ve su canlılarına olan zehirli etkisi nedeniyle çok düşük konsantrasyonlarda dahi düşüren ve insan faaliyetleri sonucu çevreye karışan maddelere özel su kirleticileri ya da kalıcı kirleticiler ya da mikrokirleticiler denilmektedir. Teknolojinin gelişmesi ile birlikte hem mikrokirleticiler, çevresel sularda daha düşük ölçüm limitlerinde ölçülebilir olmuş hem de canlı yaşamını ve ekosistem dengesini etkileyebilecek yeni kirleticiler ortaya konulmuştur. Bu tip kirleticiler henüz yönetmelikler ile denetlenmediği için görünür hale gelen anlamındaki "emerging pollutants" adı altında kategorileştirilmişlerdir. "Emerging pollutants" içerisinde yüzey aktif maddeler, ilaçlar ve kişisel bakım ürünleri sayılabilir. Bu kategorideki birçok kirletici için henüz risk değerlendirmesinde kullanılabilecek çevresel konsantrasyon ve ekotoksikolojik veriler yeterince bulunmamaktadır. Dolayısıyla bu kirleticilerin canlı yaşamına ve ekosisteme etkilerini eldeki veriler ile kestirmek çok zordur ve iyi bir değerlendirme için yeni verilere ihtiyaç bulunmaktadır.

İlaçların önemli bir olası etkisi ise maruz kalan canlıların endokrin sisteminin işlevinde yaratacağı bozuklukardır. "Endokrin sistemi bozucu" terimi ilk kez 1992'de kullanılmış olup 1996'da ABD'de bu tür maddelerin neler olabileceğine dair resmi araştırmalar yapılmaya başlanmıştır.

Önemli bir çevresel sorun olan ilaç kalıntıları Avrupa Birliği 5. Çerçeve Programı'nda araştırma önceliğine sahip alan olarak seçilmiş olup Avrupa Birliği'nin bu konudaki araştırmalara desteği 6. ve 7. Çerçeve Programları'nda da devam etmiştir.

İlaçların üretim ve kullanımları çevrede birikmelerine ve ekosistemin ilaçlardan etkilenmesine neden olur. İlaçların en önemli kaynakları hasta kullanımları sonucunda evler ve hastanelerdir. Kullanım sonrası ilaçlar vücuttan değişmeden ya da metabolit ya da konjugeleri şeklinde atılırlar.

Aıtksu arıtma tesisleri ise ilaç ve endokrin sistemi bozucu maddeleri taşıyan atıksuların toplandığı yerlerdir. Atıksu arıtma tesisleri genellikle Kimyasal Oksijen İhtiyacı deneyi ile tespit edilebilen karbonlu organik maddelerin ve azot ve fosfor gibi besi maddelerinin giderimi için tasarlanmıştırlar. Birçok ilaç biyolojik olarak

parçalanamadığı için atıksu arıtma tesisleri ilaç ve endokrin sistemi bozucu maddelerin esas kaynağı olarak kabul edilebilir.

Tarım ve hayvancılık faaliyetleri ile balık çiftlikleri ilaç ve hormonların yayılı kaynaklarıdır. Estrojenler ve diğer ilaçlar balık çiftliklerinde üremeyi artırmak için kullanılırlar. Balık çiftlikleri denizlere kuruldukları için bu tesislerde kullanılan ilaç ve hormonlar tesisin bulunduğu alanı kirletirler. Yetiştirilen hayvanlara da çeşitli ilaç ve hormonlar verilmektedir. Kullanılan bu ilaç ve hormonlar hayvan vücudundan dışkı ile atıldıktan sonra yüzeysel akış yolu ile yüzeysel sulara ulaşmaktadır.

İlaçların ve hormonların çevresel sulardaki miktarlarının belirlenmesi son derece kompleks matrislere sahip numunelerde çok hassas ölçümleri gerektirmektedir. Bu sebeple, kullanılan analitik teknikler, ölçümü yapılan maddelerin birçok safsızlıkların arasından ayrılıp belirlenmesini sağlayacak kadar spesifik, düşük ölçüm limitlerine inebilecek kadar da hassas olmalıdır. İlaçların birçoğu polar yapıda olup görece daha düşük moleküler ağırlıklara sahip oldukları için ölçümleri son derece zorlayıcı olabilir. Bu yüzden ilaçların çevresel sulardaki miktarlarının belirlenmesi ileri ölçüm tekniklerinin (GC-MS/MS, LC-MS/MS gibi) kullanımını gerektirmektedir. Hâlihazırda ilaçların ölçümünü amaçlayan metotlar literatürde yer almakta ve bu metotların sayıları hızla artmaktadır ancak yine de kullanılagelen bu analitik metotların geliştirilmesi gerekmektedir.

İlaç ve hormonların ekolojik etkileri ng/L seviyelerinde görüldüğü için ölçüm limitlerinin de bu seviyelerde olması gerekmektedir. Önceleri GC-MS ve GC-MS/MS ilaç ve hormonların kullanımları için tercih edilen ekipmanlar iken gelişen teknoloji ile hassasiyetleri artırılan ve türevlendirme gerektirmeyen LC-MS/MS sistemler bu çalışmalarda günümüzde daha sık kullanılmaktadır.

Kirleticilerin çevredeki değişim ve dönüşümlerinin belirlenmesi karmaşık bir konudur. Değişim ve taşınım prosesleri çalışılan matrise bağlıdır. Genellikle, değişim ve dönüşüm çalışmalarında iki temel yaklaşım kullanılmaktadır: laboratuvar ölçekli çalışmalar ile saha çalışmaları. Laboratuvar çalışmaları tüm proseslerin belirli bir detayı hakkında bilgi sağlarken saha çalışmaları gerçek koşullar altında, kirleticilerin davranışı hakkında açıklama yapılmasına olanak sağlarlar.

İlaçlar biyolojik bir etki yaratmak üzere tasarlandıkları için ekosistemlere ve özellikle bu ekosistemlerde yaşayan canlılara ters yönde etki edecekleri tahmin edilmektedir. Her ne kadar çevrede bulunduklarından daha yüksek dozlarda ilaçlar tedavi için kullanılsa da besin zinciri aracılığıyla ya da içme suyundan doğrudan maruz kalma ile sulardaki ilaç kalıntıları uzun maruz kalma süreleri sonucu insan sağlığını da tehdit edebilir.

Ekolojik etki belirleme çalışmalarında genellikle tek bir tür üzerinde yapılan deneyler kullanılmaktadır. Ancak bu tip deneyler türlerin etkileşimi hakkında minimum bilgi sağlamaktadır. Besin zinciri boyunca etki mekanizmasını belirlemek için bensin zincirinin farklı basamaklarında bulunan farklı türler kullanılmalıdır.

Yürürlükteki yasalara göre, yeni bir ilaç piyasaya sürülmeden önce akut ve kronik etkileri belirlenmelidir. Dolayısıyla ilaçların etkileri hakkında ilaç piyasaya sürülmeden çeşitli testler yapılır. Ancak, literatürde, ilaçların interaktif (sinerjistik/antogonistik v.b.) etkileri hakkında bazı çalışmalar olsa da bu etkiler hala yeterince ortaya konamamıştır.

Endokrin bozucu maddelerin ve ilaçların kütle tabanlı analitik cihazlar ile ölçümü kantitatif sonuçlar vermektedir. Diğer yandan daha kalitatif olan biyolojik testler ise toplam östrojenik etkiyi vermesi açısından son derece etkin araçlardır. Her iki sistemin kendine özgü avantaj ve dezavantajları ortaya konulduğu zaman hem estrojeniteyi belirlemek açısında biyolojik testlerin (YES vb.) hem de izlenen maddelerin çevresel konsantasyonlarının belirlenmesi açısından analitik ölçüm cihazlarının (LC-MS/MS) kullanımı çalışmalarda farklı bakış açıları ile değerlendirmeler yapılmasını sağlamaktadır.

Özellikle gelişmiş ülkelerde ilaç ve hormonların yüzeysel sulardaki miktarları ile ilgili çeşitli çalışmalar bulunmaktadır. Yine de ilaç ve hormonların yüzeysel sulardaki davranışlarının belirlenmesi için yeni saha çalışmalarına gereksinim duyulmaktadır.

Bu çalışmanın temel amacı sucul ortamlardaki ilaç ve hormon kalıntılarının varlığı, değişimi ve etkileri üzerine bilimsel bilgi oluşturmaktır. Çok fazla kullanılan 10 adet ilaç etken maddesi (3 adet steroid olmayan ateş düşürücü, diklofenak, ibuprofen, naproksen; 4 adet antibiyotik, amoksisilin, siprofloxasin, eritromisin ve sulfametoksazol; 2 adet beta bloker, atenolol ve propranolol ve bir adet uyarıcı, kafein) ve 4 adet östrojen hormon estrone (E1), estradiol (E2), estriol (E3), ve 17α-ethynylestradiol (EE2) bir yıllık satış verilerine gore seçilmiştir. Seçilen ilaç ve hormon kalıntılarının İstanbul'da bulunan bir yüzeysel sudaki varlığı araştırılmıştır.

Önemli bir içme suyu kaynağı olan Büyükçekmece Gölü ve bu göle akan beş adet derede seçilen ilaçların anlık konsantrasyonları izlenmiştir. Mevsimsel değişimleri izlemek amacıyla yılın beş farklı zamanında numune alınmıştır. Katı faz ekstraksiyonu ve tandem kütle spektroskopisine bağlı ultra performanslı sıvı kromatograf kullanılarak hızlı ve hassas bir ölçüm yöntemi geliştirilmiştir. Maddelerin polarite farklarından dolayı literatürde genellikle ilaç ve hormonlar için ayrı yöntemler bulunmaktadır. Geliştirilen numune hazırlama yöntemi ile ilaç ve hormonların tek bir ölçüm yöntemi kullanılarak ölçülmesini olanaklı kılmıştır.

Geliştirilen bu yöntemde farklı maddeler için 0,5 ila 1,1 ng/L arasında en düşük ölçüm limitleri elde edilmiştir. Ultra saf su ve yüzeysel su için sırasıyla %72-119 arasında ve %61-98 arasında geri kazanımlar elde edilmiştir. Bazı ilaçlar, nehirlerde µg/L seviyesinde ölçülmüştür. En sık tespit edilen maddeler kafein ve antibiyotikler iken sentetik bir hormon olan EE2 sadece 4 kez tespit edilerek en az tespit edilen madde olmuştur.

İlaç ve hormonların ekolojik etkilerini belirlerken besin zincirinde farklı yerlerde bulunan canlılar üzerindeki farklı etkiler hakkında bilgi edinebilmek üzere çeşitli ekotoksikolojik araçlar kullanılmıştır. *P. subcapitata* akut ekotoksik etkileri belilemek üzere kullanılırken *D. magna* hem akut hem de kronik ekotoksik etkilerin belirlenmesinde kullanılmıştır. Mutajenik ve östrojenik etkileri belirlemek üzere sırasıyla AMES ve YES testleri kullanılmıştır.

P. subcapitata ve *D. magna* ile yürütülen deneyler sonucunda ilaç ve hormonların yüzeysel sularda bulunan konsantrasyonlarının herhangi bir akut etki yaratması beklenmese de çalışılan maddelerin kronik etkilerinin ekosistem dengesini sarsıcı olabileceği gözlenmiştir.

Tekil maddelerin etkilerinin gözlenmesinin yanında, bilimsel literatürde bulunan bilgi eksikliği nedeniyle ilaç ve hormon karışımlarının yarattığı etkiler de çalışılmıştır. *D. magna* akut ve kronik ve *P. subcapitata* akut ekotoksisite testlerinde

bütün karışımlar sinerjistik etki göstermiştir. Ayrıca maddelerin tekil olarak etki göstermedikleri konsantrasyonları karıştırılmaları durumunda bu testlerde ekotoksik etki yaratmaktadırlar. Bu sonuçlar, tekil maddelerin çevresel risk değerlendirme çalışmalarında kullanılan eşik değerlerinin yanında interaktif etkilerinin de belirlenmesinin daha anlamlı ve yararlı olacağını göstermektedir.

1. INTRODUCTION

Definition of water pollution caused by chemical substances has long a history. In 1954, W. Haynes in his 6 volume book, American Chemical Industry – A History, wrote that "by sensible definition any by-product of a chemical operation for which there is no profitable use is a waste. The most convenient, least expensive way of disposing of said waste – up the chimney or down the river – is the best." It is clear that once anything other than product, particularly wastes had been removed from industrial facilities without considering ecosystem integrity and human health (Hemond and Fechner-Levy, 2000).

After the mid-60s people have become aware that some substances –for instance mercury derivatives (i.e., methyl mercury), DDT and PCBs- can persist in the environment, enter and became enriched in food chains and reach toxic levels in certain organisms. Rachel Carson's book "Silent Spring" played an important role on this awareness and "enlightenment" on toxic substances. The recognition of toxicity has been a driving force behind the development towards better environmental management and stricter regulations. Furthermore, studies triggered by the recognition of these effects led to realization that anthropogenic contaminants are present everywhere in the environment, and many of these substances are potentially hazardous to ecosystem.

To emphasize the difference between identifiable chemical substances and classical aggregate or general parameters such as Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD) and suspended solids, the concept of "specific water pollutant" is being used. The specific water pollutant was defined, by Organisation for Economic Co-operation and Development (OECD), as a substance which is mainly introduced into the environment by human activity and which, under given conditions, lowers the quality and value of a water resource, particularly by toxic and nuisance effects on human beings or aquatic life (Organisation for Economic Co-operation and Development., 1982). Specific water pollutants have also been called "trace pollutants", "micropollutants", and "refractory pollutants".

With the development of technologically advanced analytical techniques, micropollutants have become a popular study area and scientists divided micropollutants into further classes. For instance, the class of "Emerging contaminants" corresponds in most cases to unregulated contaminants, which may be candidates for future regulation depending on the results of research on their potential health effects and available monitoring data regarding their occurrence. Emerging pollutants include several groups of compounds such as surfactants, pharmaceuticals, and personal care products (PPCPs) and gasoline additives. Although these groups of compounds may have low half-lives in the environment, their continuous release may lead to accumulation in the environment and hence cause adverse effects. It is difficult to predict effects of emerging contaminants since there are gaps on data on their occurrence and ecotoxicological effects (Petrovic et al., 2003). The risk assessment is particularly difficult to conduct since the presence of these compounds in mixtures might lead to significantly different effects compared to their effect as a single compound.

One possible effect of pharmaceuticals is their effect on the endocrine system of the exposed organisms. The term "endocrine disrupter" gained popularity in 1992 when Colborn and Clement used it to address negative effects of foreign chemicals to endocrine system. In 1996, The U.S. Environmental Protection Agency (USEPA) initiated studies to identify endocrine disrupting effects of chemicals and classify them. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was founded for this purpose (Cline, 2002). In 1998, the EDSTAC defined the endocrine disrupters as "an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its pyrogenity, populations, or subpopulations of organisms, based on scientific principles, data weight of evidence, and the precautionary principle" (EDSTAC, 1998). Meanwhile, National Research Council (NRC) of USA defined the endocrine disruption of chemicals as hormonally active agents (HAA). HAA was defined regardless to the specific mode or mechanism of action of the chemical to expand the issue. However, the term, hormonally active agents, has not gained popularity compared to the term, endocrine disruptors, among the public or scientific community (Cline, 2002).

As an emerging environmental issue, pharmaceutical residues in the environment was selected as a research priority in the European Union 5th Framework Programme (Ternes and Joss, 2006) and the attention of European Union to this issue continued during 6th Framework Programme, as well.

The production and use of pharmaceuticals lead to a potential environmental exposure and also to an accumulation in certain environmental compartments. The main discharge routes of human pharmaceuticals to the environment are expected to be through their use by patients in private households, in hospitals and the subsequent disposal of these pharmaceuticals through toilets. After their use, pharmaceuticals are excreted as unchanged compound and/or metabolites in feces and urine and hence are present in wastewater (Halling-Sorensen et al., 1998).

Wastewater treatment plants are placed downstream of sewer systems carrying pharmaceuticals and endocrine disrupters. Wastewater treatment plants were usually designed to treat carbonaceous organic matter which can be measured as COD and also nutrients such as nitrogen and phosphorus. Many pharmaceuticals are relatively resistant to degradation in these wastewater treatment plants and therefore, wastewater treatment facilities are the major sources for pharmaceuticals and endocrine disrupters together with industrial processes that use cleaners and plastics (Golet et al., 2001; Routledge and Sumpter, 1996; Snyder et al., 2003; Staples et al., 1998; Sumpter, 1995; Ternes and Hirsch, 2000; Ternes and Joss, 2006; Ying et al., 2002).

Agriculture, livestock feed and fish farms have been identified as non-point sources of pharmaceuticals and hormones. Estrogens and some other pharmaceuticals are used in fish farms to increase productivity. Since fish farms are located in marine environment, pharmaceuticals and hormones that are used in these fish farms may easily contaminate the area. Livestock is generally administered pharmaceuticals and hormones, as well, which are excreted in manure and urine. All excreted pharmaceuticals and hormones can easily reach to surface water via agricultural runoff (Campbell et al., 2006; Kolodziej et al., 2004).

Although it importance of and the need for occurrence studies in surface water, and in particular in drinking water sources is clear and well understood, the measurement of pharmaceuticals and hormones in surface water is quite challenging, since it

requires technologically advanced analytical equipment. Moreover, the analytical methods have to be specific and sensitive enough to eliminate possible interferences in complex matrices and to quantify target compounds down to ng/L levels. Therefore, the ultimate analytical method should include an efficient enrichment technique such as solid phase extraction (SPE) and modern separation and detection techniques including gas chromatography coupled with mass spectrometer (GC-MS, GC-MS/MS) and liquid chromatography coupled with mass spectrometer (LC-MS, LC-MS/MS)

Since ecological effects of pharmaceuticals and hormones are observed at concentrations as low as ng/L, achievement of low analytical detection limits is required. GC-MS and/or GC-MS/MS used to be the method of choice for the measurement of PCPP and hormones. However, due to developments in sensitivity of LC-MS/MS leading to lower detection limits as well as the lack of the need for a derivatization step for most of the compounds, LC-MS/MS recently has started to be used more frequently. Currently, the number of available analytical methods for the detection of pharmaceuticals and endocrine disrupting compounds is increasing but many still need to be developed for complex matrices (Fatta et al., 2007; Ternes and Joss, 2006).

The determination of the environmental fate of a compound is a complex issue. Transformation and distribution processes are strongly dependent on the specific environmental conditions (Figure 1.1). In general, there are two major approaches for environmental fate studies: Laboratory and field studies. Field studies allow for the elucidation of substances behavior under realistic conditions, whereas laboratory experiments display only certain details of the entire scenario (Ternes et al., 2005).

Since pharmaceuticals are designed with the intention of a biological effect, ecosystems, particularly organisms living in those ecosystems, might be adversely affected by unchanged pharmaceuticals and their metabolites discharged to the environment. Researches indicate that some pharmaceuticals (e.g., ethynylestrodiol used in hormone replacement therapy) affect endocrine system of organisms in concentration levels as low as ng/L (Sumpter and Jobling, 1995). Although higher pharmaceuticals are used at high concentrations for a short time, these compounds may also threat human health via both food web and direct exposure through drinking water, especially when more than one pharmaceutical is present and the

duration of exposure is long compared to the duration of intended use for a disease. Therefore, pharmaceuticals in ecosystems, in particular, in drinking water resources must be monitored and their fate and transport mechanisms and effects should be identified in order to take action against possible adverse effects of pharmaceuticals and to protect human health.

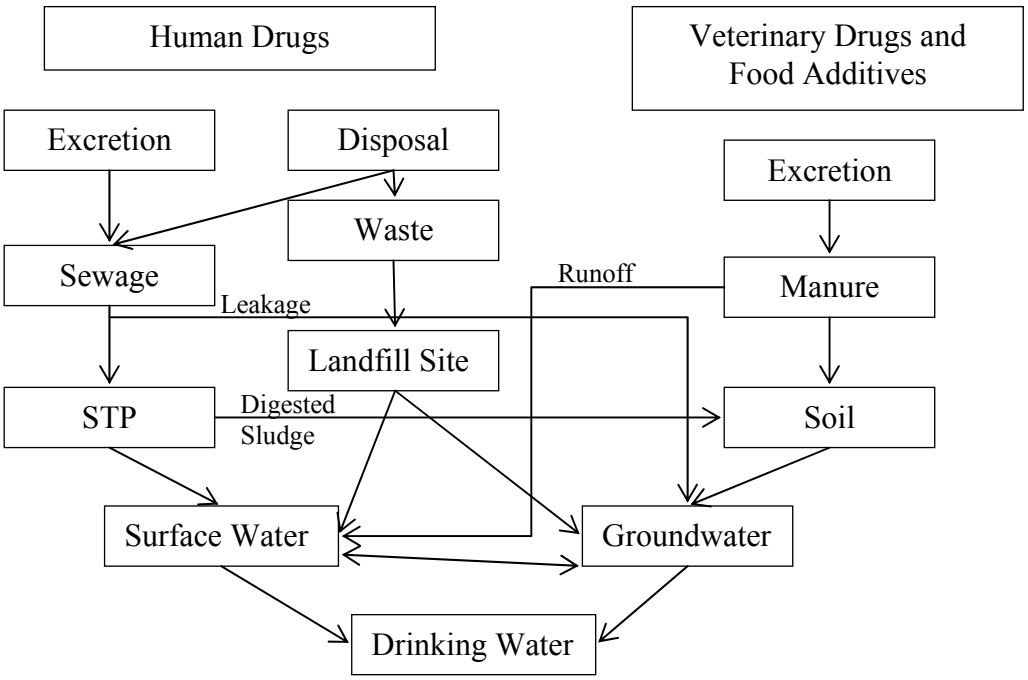


Figure 1.1: Fate and transport of pharmaceuticals in the environment (Ternes, 1998).

Generally, single species tests are used as bioassays in ecological impact assessment studies. Single species tests are standard, informative and may provide a great deal of information. However, they provide only minimal information on species' interactions. Therefore, different species at different levels of the food web should be selected to understand the effect mechanisms of a compound through food chain. Moreover, *in-vitro* tests also provide important data on sub-lethal and sub-chronic effects (Hodgson, 2004).

Before introduction of a new medicine to the market, acute and chronic effects should be identified according to EU and US legislations. Consequently, there will be information on effects of new medicines. However, lack of knowledge on interactive effects (synergistic/antagonistic/potentiation) of pharmaceuticals still goes on (Santos et al., 2010). Moreover, single ecotoxicological effects are concerned during environmental risk assessment studies although pharmaceuticals and

hormones do not present as single compounds in the environment (Jesus Garcia-Galan et al., 2008; Kolpin et al., 2002; la Farre et al., 2008). Some studies indicate that mixture effects of pharmaceuticals might be different than effects of single compounds (Cleuvers, 2003; DeLorenzo and Fleming, 2008; Quinn et al., 2008). Still these studies are far from filling knowledge gaps on mixture toxicity (Santos et al., 2010).

As mentioned previously, quantitative analysis of pharmaceuticals and hormones can be conducted with mass spectrometers. On the other hand, bioassays are essential tools for qualitative analysis for determination of the effects of pharmaceuticals and hormones, such as endocrine disruption. Considering advantages and disadvantages of both of the systems, the use of bioassays (*e.g.,* YES) to determine the impacts and the analytical techniques (*e.g.,* LC-MS/MS) to obtain occurrence data, may provide different angles to make better assessments on the fate of pharmaceuticals and hormones (Heisterkamp et al., 2004).

Numerous studies are conducted in developed countries reporting the occurrence of pharmaceuticals and hormones in surface waters. In these studies, pharmaceuticals and hormones ranged from ng/L level to µg/L level (Castiglioni et al., 2005; Feitosa-Felizzola and Chiron, 2009; Fernandez et al., 2010; Gracia-Lor et al., 2011; Gros et al., 2006; Kasprzyk-Hordern et al., 2008c; Kleywegt et al., 2011; Kuster et al., 2008; Vanderford et al., 2003; Watkinson et al., 2009). Nevertheless, more field data are required on the effects and fate of pharmaceuticals in the aquatic environment (European Environment Agency, 2010). Besides, since concentrations of pharmaceuticals in wastewaters and surface water depend on the water and pharmaceutical usage rates, it may not be possible to estimate PPCP concentrations in one region based on studies conducted in other regions of the world, especially when the extrapolation is conducted between developed and developing/underdeveloped countries.

1.1 Aim and Scope

The aim of this study is to provide specific information on the occurrence, fate, and effects of pharmaceuticals and hormones in aquatic environment. For this purpose, Büyükçekmece Watershed encompassing an important drinking water source of Istanbul was selected to conduct field studies. 14 widely used pharmaceuticals in

Turkey and hormones were selected according to one year-sales data obtained from IMS Health Turkey, in this study.

Analytical methods for the measurement of pharmaceuticals and hormones in the environment need to be specific enough for the detection of target compounds among numberless impurities and interferences and sensitive enough to achieve low quantification limits. Although several methods are available in the literature, the measurement method should be developed specifically for each analytical equipment. Therefore, in this study, a rapid and sensitive detection and quantification method using an ultra-performance liquid chromatograph coupled with a triple quadrupole mass spectrometer (UPLC-MS/MS) was developed for 14 pharmaceuticals and hormones. The method consists of a SPE phase for enrichment of selected compounds as well as removal of interferences and a detection phase with UPLC-MS/MS. This method was applied in order to monitor selected compounds in Büyükçekmece Watershed to enable future environmental and human health risk assessment studies.

During occurrence studies, samples were taken from Büyükçekmece Lake and its 5 main tributaries. The effect of seasons was also captured by taking samples five different times in a year.

Different tools were used for the determination of ecological impacts of selected pharmaceuticals and hormones to cover different effects and understand responses of different species at different levels of the food web. *P. subcapitata* was used for the determination of acute effects whereas *D. magna* was used for the determination of both acute and chronic effects. Mutagenic and endocrine disruptive effects were determined with AMES and YES test, respectively. Moreover, since the effects of compounds in mixtures are not necessarily the same as the effects of single compounds, effects of selected compounds were also determined in mixture which are formed based on occurrence data.

1.2 Main Findings

A rapid and sensitive analytical measurement method was developed for measuring pharmaceuticals and hormones in surface water. Since polarities of pharmaceuticals and hormones are different, available analytical methods tend to measure them

separately. The developed method is one of the few in the literature for multi-residue analysis of both pharmaceutical and hormones.

This is the first study in Turkey and one of the few studies in developing countries reporting the occurrence of pharmaceuticals and hormones in surface water. Therefore, this study provides valuable information for future environmental and human health risk assessment studies. Moreover, ecotoxicological data on mixture effects provide valuable information to understand interactive effects of pharmaceutical and hormones on which there is a huge knowledge gap worldwide.

Since occurrence data are the results of a field study conducted in a watershed used for the supply of drinking water in Istanbul, they provide information for decision makers to take action against possible adverse effects of studied compounds. Moreover, the data will be helpful for the implementation of Water Framework Directive of European Commission.

2. LITERATURE REVIEW

2.1 Consumption of Pharmaceuticals and Hormones

Generally, there is a positive correlation between the most frequently used classes of pharmaceutical and their detection in the aquatic environment. Many of the top sold pharmaceuticals are specific beta blockers, lipid regulators, antidiabetic, antianginal drugs, as well as analgesics and antibiotics (Jones et al., 2001). It is estimated that 100,000 tons per year of pharmaceuticals are consumed in global scale corresponding approximately 15 g/cap.year (Kümmerer, 2004). Personal consumption may increase to 150 g/cap.year in developed countries (Ternes and Joss, 2006). Estimation of consumption of pharmaceuticals is a controversial issue. There are different methods to collect data. It may rely on prescriptions or sales. It is well known that non-prescribed sales of pharmaceuticals at least ten times higher than prescribed sales (Kümmerer, 2004). Therefore, consumption of pharmaceuticals and hormones must be estimated according to sales data. However, it is difficult to achieve sales data of pharmaceuticals in particular developing countries such as Turkey.

Trends and habits of consumption of pharmaceuticals and hormones may differ from country to country and over time. In general, non-steroidal anti-inflammatory drugs (NSAID) and antibiotics are mostly consumed pharmaceuticals all over the world. However, it is known that consumption of pharmaceuticals may differ from country to country even said pharmaceuticals belong to the same therapeutic group. For instance, while ibuprofen the most consumed NSAID in Sweden it is diclofenac for Austria (Ternes and Joss, 2006). Moreover, antibiotics are consumed more in developing countries than in developed countries. Furthermore, antibiotics and analgesics are consumed more in winter.

From now on there is only one study conducted in Turkey that predicts environmental concentrations of only antibiotics using PEC/PNEC model (Turkdogan and Yetilmezsoy, 2009).

2.2 Occurrence of Pharmaceuticals and Hormones in Aquatic Environment

There are different routes that pharmaceuticals and hormones enter to the environment. Sewer systems are the main collection structures of pharmaceuticals and hormones since after usage or flushed down from toilets in households or hospitals, they enter to sewer systems and eventually end up in a wastewater treatment plant. In many research, it is observed that many pharmaceuticals are resistant towards degradation during wastewater treatment and they are discharged to environment via treated wastewater from wastewater treatment plants which may be considered as main sources of pharmaceuticals (Golet et al., 2001; Routledge and Sumpter, 1996; Snyder et al., 2003; Staples et al., 1998; Sumpter and Jobling, 1995; Ternes and Hirsch, 2000; Ternes and Joss, 2006; Ying et al., 2002). There is also possibility that discharge of untreated wastewater to surface water in developing countries such as Turkey. There are other minor routes of pharmaceuticals and hormones to the environment as: release of private septic/leach fields, reinjection to aquifers or reuse for irrigation of treated wastewater, transfer of biosolids to land, release from agriculture, manure from medicated domestic animals, direct release to open water via washing/bathing/swimming, discharge of controlled industrial wastewater, disposal from illegal drug labs and illicit drug usage, disposal to landfills via domestic refuse and medical waste, leaching from landfills and cemeteries, release to open water from aquaculture,. Moreover, ultimate fate and transport mechanisms of pharmaceuticals and hormones in the environment may cause further release (Petrović and Barceló, 2007). Therefore, pharmaceuticals may accumulate certain points in the environment and living organisms after use and disposal (Halling-Sorensen et al., 1998).

Approximately 10-100 µg/day estrogens are removed from a woman's body in normal menstrual cycle. This amount may increase to 30 mg/day during pregnancy. Most of the estrogenic activity of wastewater and surface water is caused by E2 and EE2 in ng/L concentrations (Snyder et al., 2001). Although predicted no effect concentration (PNEC) of EE2 was estimated as 0.35 ng/L (Skotnicka-Pitak et al., 2008), it is observed that 0.1 ng/L EE2 had been triggered feminization of fish (Purdom et al., 1994). Nevertheless, other natural hormones, E1 and E3, go into wastewater via urine and are expected to have endocrine disruptive effect since they have similar metabolites with E2 and EE2.

Mass spectrometric methods are used to determine occurrence and fate of pharmaceuticals and hormones in the environment. Although these methods do not provide information on estrogenic activity of target compounds, their sensitivity and selectivity make them essential for quantification of compounds as low as ng/L levels (Campbell et al., 2006).

Mass spectrometer (MS) is used as hyphenated technique to both of the gas chromatographer (GC) and liquid chromatographer (LC). MS is not only very sensitive and selective but also provides information about molecular structure of measured compounds. The only available method to quantify organic materials in complex environmental matrices is MS. Before, GC-MS was generally used to quantify organic compounds thanks to its very high chromatographic resolution. However, GC had actually been designed to quantify volatile and half volatile organic compounds. After development of electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) in 90s, LC-MS started to be used more commonly. High resolution or tandem MS (HRMS or MS/MS) provide detailed structural information and in most cases are necessary to quantify pharmaceuticals and hormones at low concentrations since they make possible identification of compounds having same molecular weight even though they do not chromatographically separated (Fatta et al., 2007; Ternes and Joss, 2006).

Analytical techniques used for measurement of rather more polar and low molecular weighted pharmaceuticals and hormones in environmental waters must be sensitive and selective enough to make quantification possible down to ng/L levels. Therefore, technologically advanced hyphenated analytical techniques (*e.g.,* GC-MS/MS and LC-MS/MS) must be used in multi-residue analysis of pharmaceuticals and hormones in environmental waters. One disadvantage of these kinds of analytical techniques is need of long time to achieve reliable determination method. Although there are some analytical measurement methods for quantification of pharmaceuticals and hormones in the literature, there is still need for development of new methods in particular for complex water matrices (Ternes and Joss, 2006).

Although GC methods are very sensitive and selective, their need for derivatization of polar and charged compounds diverts the attention to LC methods (Ternes and Joss, 2006).

Ternes (2001) directly compared GC-MS and LC-electrospray ionization (ESI)-MS/MS, and showed that only LC-(ESI)-MS/MS allows the analysis of extreme polar compounds (e.g., b-blockers, atenolol and sotalol) due to an incomplete derivatization of the functional groups. Further, the relative standard deviation using LC-(ESI)-MS/MS was found to be lower. However, when analyzing highly contaminated samples, such as sewage, suppression of electrospray ionization is likely to occur, so, to guarantee accurate, reproducible data, either an efficient clean-up step has to be included in sample preparation or an appropriate surrogate standard has to be spiked prior to enrichment by solid phase extraction (SPE).

Farré et al. (2001) compared LC-(ESI)-MS and GC-MS (after derivatization with BF3-MeOH) for monitoring some acidic and very polar analgesics (salicylic acid, ketoprofen, naproxen, diclofenac, ibuprofen, and gemfibrozil) in surface water and wastewater. The results showed a good correlation between methods, except for gemfibrozil, for which derivatization was not completely achieved in some samples. In general, the limits of detection (LODs) achieved so far with LC-MS/MS methods are slightly higher than those obtained with GC-MS methods; however, LC-MS methodology showed advantages in terms of versatility and sample preparation being less complicated (i.e. derivatization is not needed) (Diaz-Cruz and Barcelo, 2005; Farré et al., 2001). Since there are various advantages of LC-MS/MS methods against GC-MS/MS methods and the determination and measurement of concentrations of most of the pharmaceuticals are possible with LC-MS/MS (Figure 2.1), LC-MS/MS became more popular in scientific community for measurement of pharmaceuticals and hormones in environmental waters.

During analysis of pharmaceuticals and hormones, an enrichment method should be used to reach ng/L levels. Solid phase extraction (SPE) is the most widely used enrichment technique (Ternes and Joss, 2006). Previously, C18 was used as solid phase for enrichment of pharmaceuticals and hormones. Then other SPE cartridges having engineered adsorbents such as EnciCarb, LiChrolut, Isolut ENV+, Oasis HLB, and Oasis MCX started to be used. Recently, Oasis HLB is cartridge of choice in most of the studies thanks to its hydrophilic and lipophilic balanced adsorbent increasing recoveries and adsorbing nearly all of the pharmaceuticals and hormones.

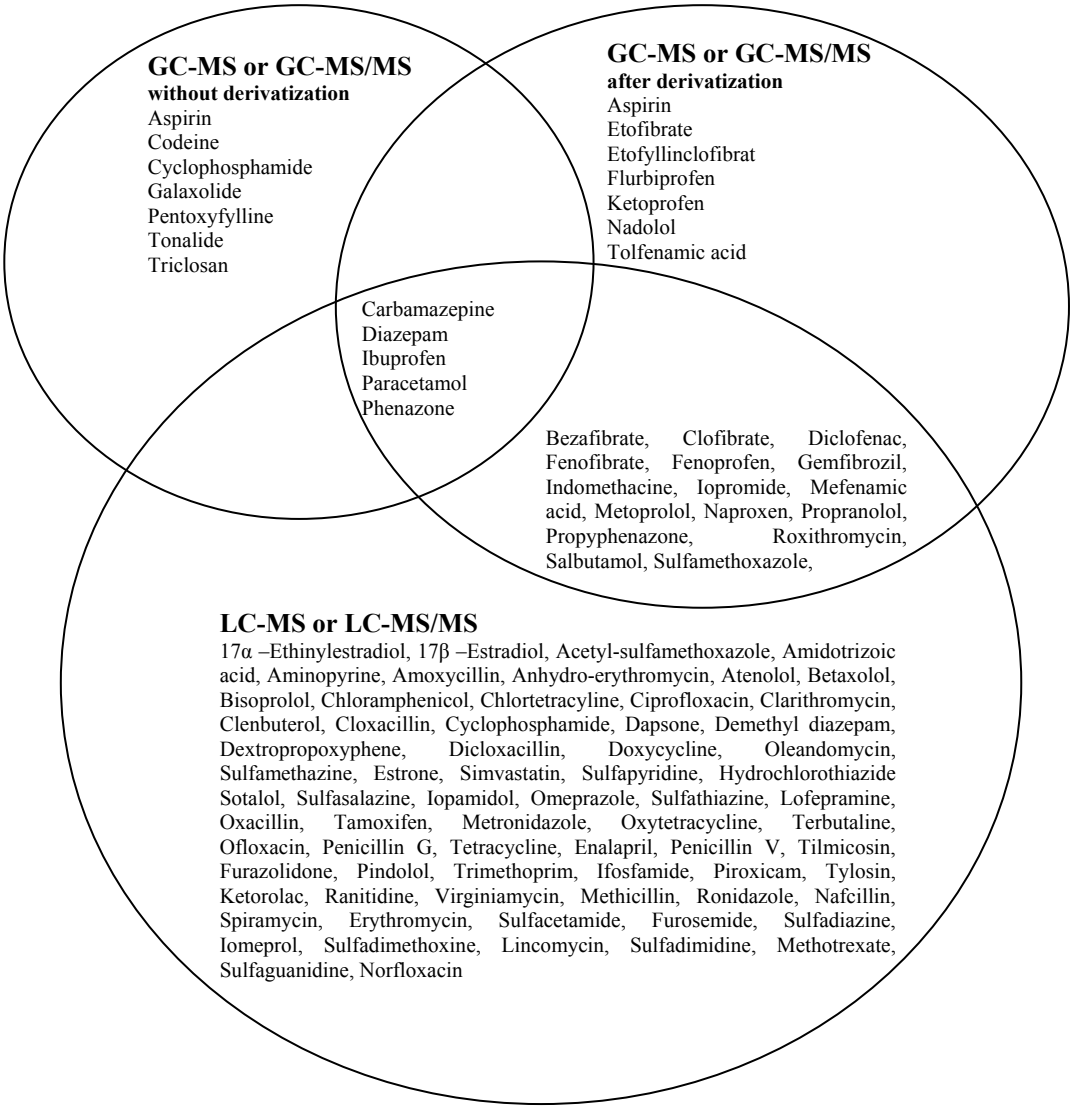


Figure 2.1: Analytical methods applied for the most common pharmaceuticals in water and wastewater (Fatta et al., 2007).

Today most of the studies in the literature are on antibiotics, NSAIDs, and blood lipid lowering agents due to their high prescription rates. Studies on sex hormones and β-blockers come next (Santos et al., 2010). Occurrence studies generally focus on pharmaceuticals and hormones in surface water and wastewater. There are small amount of studies on pharmaceuticals and hormones in groundwater and sediment.

Antibiotics generally measured at low ng/L concentrations in surface water (Table 2.1).

Table 2.1: Literature survey on concentrations of studied antibiotics in surface water.

Compound	Sample	Country	Analytical Procedure	LOD (ng/L)	Measured Concentration (ng/L)	Reference
Amoxicillin	Surface water	UK	LC-MS/MS	10	ND-552	(Kasprzyk-Hordern et al., 2008c)
Ciprofloxacin	Surface water	USA	LC-MS	20	20	(Kolpin et al., 2002)
	Po River	Italy	LC-MS/MS	0.3	ND-26.15	(Calamari et al., 2003)
	Lambro River	Italy	LC-MS/MS	0.3	1.4-15.90	(Calamari et al., 2003)
	Mankyung River	South Korea	LC-MS/MS	1	ND-137	(Kim et al., 2009)
Erythromycin	Victoria Harbour Seawater Pearl River Water	China	LC-MS	2 (LOQ seawater) 5(LOQ river water)	5.1-6.1	(Xu et al., 2007)
	Surface water	USA	LC-MS	50	150	(Kolpin et al., 2002)
	Drinking water	USA	LC-MS/MS	0.25	0.32	(Benotti et al., 2009)
	Alzette River	Luxembourg	LC-MS/MS	0.3	1-22	(Pailler et al., 2009)
	Mess River	Luxembourg	LC-MS/MS	0.3	0.3-5	(Pailler et al., 2009)
Sulfamethoxazole	Surface water	UK	LC-MS/MS	0.5	ND-351	(Kasprzyk-Hordern et al., 2008c)
	Surface water	South Korea	LC-MS/MS	1	1.7-36	(Kim et al., 2007a)
	Han River	South Korea	LC-MS	5	ND-82	(Choi et al., 2008)
	Rio Grande River	USA	LC-MS	12	ND-300	(Brown et al., 2006)
	Tevere River	Italy	LC-MS	9	402	(Perret et al., 2006)
	Drinking Water	Italy	LC-MS	9	13-80	(Perret et al., 2006)
	Pearl River	China	LC-MS	1 (LOQ)	37-134	(Xu et al., 2007)

LOD: Limit of Detection
LOQ: Limit of Quantification
ND: Not Detected

All of the studies on occurrence of studied antibiotics in surface water were conducted in developed countries. There is not any concentration pattern for each antibiotic. Concentrations are ranged from <0.3 to 550 ng/L.

There are more studies on occurrence of antibiotics in wastewater. Obviously concentrations in wastewater were measured higher than concentrations in surface water. Ciprofloxacin was measure as high as 1000 ng/L and 300 ng/L in influent and effluent of wastewater treatment plant, respectively (Brown et al., 2006; Lindberg et al., 2005; Seifrtova et al., 2008). Moreover, in ciprofloxacin concentration was once reported as 11 µg/L in hospital wastewater (Seifrtova et al., 2008). Although similar concentrations were reported for erythromycin in influent between 226 and 1537 ng/L, effluent concentrations were measured higher than ciprofloxacin between 361 and 811 ng/L (Lin et al., 2009). One of the most studied antibiotics, sulfamethoxazole, was in different ranges in different countries. For instance, while concentration in influent between 179 and 1760 ng/L and in effluent between 47 and 964 ng/l in Taiwan (Lin et al., 2009), in Luxemburg, the concentration in influent was reported between 13 and 155 ng/L and in effluent between 4 and 39 ng/L (Pailler et al., 2009).

β-blockers are the least studied compounds among the compounds in this study (Table 2.2).

There is a big difference of highest reported concentration of atenolol in surface water between South Korea and other countries.

Atenolol was measured as high as 2883 ng/L, 1168 ng/L, 800 ng/L in the influent of wastewater treatment plant in Taiwan, Italy, and Finland, respectively (Castiglioni et al., 2005; Lin et al., 2009; Vieno et al., 2006). 440 ng/L effluent concentration in Finland and 681 ng/L effluent concentration in Taiwan indicate low removal efficiency of atenolol. In one case atenolol was reported 122 µg/L in hospital wastewater in Spain (Gomez et al., 2006).

Table 2.2: Literature survey on concentrations of studied β-blockers in surface water.

Compound	Sample	Country	Analytical Procedure	LOD (ng/L)	Measured Concentration (ng/L)	Reference
Atenolol	Vantaa River and Luhtajoki River	Finland	LC-MS/MS	11.8	11.8-25	(Vieno et al., 2006)
	Hoje River	Sweden	LC-MS/MS	NA	10-60	(Bendz et al., 2005)
	Po River and Lambro River	Italy	LC-MS/MS	0.3	3.44-39.43	(Calamari et al., 2003)
	Drinking water	USA	LC-MS/MS	0.25	0.47	(Benotti et al., 2009)
	Mankyung River	South Korea	LC-MS/MS	30	ND-690	(Kim et al., 2009)
Propranolol	Hoje River	Sweden	LC-MS/MS	NA	ND-10	(Bendz et al., 2005)
	Tyne River	UK	LC-MS/MS	10	35-107	(Roberts and Thomas, 2006)
	Mankyung River	South Korea	LC-MS/MS	10	ND-40.1	(Kim et al., 2009)
	Surface water	UK	LC-MS/MS	10	ND-37	(Hilton and Thomas, 2003)

LOD: Limit of Detection
LOQ: Limit of Quantification
ND: Not Detected
NA: Not reported

Since propranolol's excretion rate as unchanged compound is below 1%, concentrations of propranolol rather low. Even in wastewater, propranolol was measured 50 ng/L, 119 ng/L, and 180 ng/L in Sweden and UK (Bendz et al., 2005; Hilton and Thomas, 2003; Roberts and Thomas, 2006).

Estrogens were measured in low ng/L concentrations (Table 2.3).

Table 2.3: Literature survey on concentrations of studied hormones in surface water.

Compound	Sample	Country	Analytical Procedure	LOD (ng/L)	Measured Concentration (ng/L)	Reference
E1	Alzette River	Luxembourg	LC-MS/MS	0.3	0.3-6	(Pailler et al., 2009)
	Mess River	Luxembourg	LC-MS/MS	0.3	0.3-27	(Pailler et al., 2009)
	Tamagawa and Kasumigura Rivers	Japan	LC-MS/MS	0.1	3.4-6.6	(Isobe et al., 2003)
	Surface water	Germany	LC-MS/MS	0.1	0.16	(Zuehlke et al., 2005)
	Tibre River	Italy	LC-MS/MS	0.1	5-12	(Lagana et al., 2004)
	Surface water	France	LC-MS/MS	0.02	0.3	(Vulliet et al., 2008)
E2	Alzette River	Luxembourg	LC-MS/MS	1	1-35	(Pailler et al., 2009)
	Mess River	Luxembourg	LC-MS/MS	1	1-6	(Pailler et al., 2009)
	Tamagawa and Kasumigura Rivers	Japan	LC-MS/MS	0.3	0.6-1.0	(Isobe et al., 2003)
	Surface water	Germany	LC-MS/MS	0.2	ND	(Zuehlke et al., 2005)
	Tibre River	Italy	LC-MS/MS	0.2	2-6	(Lagana et al., 2004)
E3	Surface water	South Korea	LC-MS/MS	5	ND	(Kim et al., 2007a)
	Tibre River	Italy	LC-MS/MS	0.1	2-5	(Lagana et al., 2004)
EE2	Alzette River	Luxembourg	LC-MS/MS	2	ND	(Pailler et al., 2009)
	Mess River	Luxembourg	LC-MS/MS	2	ND	(Pailler et al., 2009)
	Surface water	South Korea	LC-MS/MS	1	ND	(Kim et al., 2007a)
	Surface water	Germany	LC-MS/MS	0.2	ND	(Zuehlke et al., 2005)
	Tibre River	Italy	LC-MS/MS	0.4	ND-1	(Lagana et al., 2004)

LOD: Limit of Detection
LOQ: Limit of Quantification
ND: Not Detected

Estrogen hormones were reported at the lowest concentrations among studied compounds in the literature.

Even though low concentrations of E1 in surface water 197 ng/L and 110 ng/L in influent and effluent of wastewater treatment plant in Japan were reported (Nakada et al., 2006). High concentration of E1 in influent of wastewater treatment plant was also reported in Germany as 188 ng/L (Zuehlke et al., 2005).

Concentrations of E2 in influent of wastewater treatment plant was similar to surface water in in Japan, Italy and Germany with concentration range 10-31 ng/L (Lagana et al., 2004; Nakada et al., 2006; Zuehlke et al., 2005). Higher concentration range was reported in Luxemburg for E2 in influent as 1-102 ng/L (Pailler et al., 2009). In effluent much lower concentrations (0.49-12.4 ng/L for Japan, 0.8 ng/L for Germany, and 2-6 ng/L for Italy) were observed.

Similar effluent concentrations for E3 were reported in Japan (0.31-0.84 ng/L), South Korea (8.9-25 ng/L), and Italy (<0.5-1 ng/L) (Kim et al., 2007a; Lagana et al., 2004; Nakada et al., 2006).

Below detection limit concentration was generally reported for EE2 in effluents except in South Korea (1.3 ng/L) and Germany (1.7 ng/L).

After antibiotics, NSAID is the most studied and most detected pharmaceutical group (Santos et al., 2010). Therefore, there are more occurrence data reported (Table 2.4).

Table 2.4: Literature survey on concentrations of studied NSAIDs in surface water.

Compound	Sample	Country	Analytical Procedure	LOD (ng/L)	Measured Concentration (ng/L)	Reference
Diclofenac	Hoje River	Sweden	GC-MS	NA	10-120	(Bendz et al., 2005)
	Paraiba do Sul River	Brazil	GC-MS	10	20-60	(Stumpf et al., 1999)
	River water	Germany	LC-MS/MS	7	26-72	(Hernando et al., 2006)
	Elbe River and Alster Lake	Germany	GC-MS	0.08	42-67	(Weigel et al., 2004)
	Alzette River	Luxembourg	LC-MS/MS	0.3	0.3-55	(Pailler et al., 2009)
	Mess River	Luxembourg	LC-MS/MS	0.3	0.3-19	(Pailler et al., 2009)
	Surface water	South Korea	LC-MS/MS	1	8.8-127	(Kim et al., 2007a)
	Surface water	UK	LC-MS/MS	20	350-460	(Hilton and Thomas, 2003)
Ibuprofen	Somes River	Romania	GC-MS	30	ND-115	(Moldovan, 2006)
	Hoje River	Sweden	GC-MS	NA	10-220	(Bendz et al., 2005)
	Po River	Italy	LC-MS/MS	4.2	ND-9.76	(Calamari et al., 2003)
	Lambro River	Italy	LC-MS/MS	4.2	78.5	(Calamari et al., 2003)
	Tyne River	UK	LC-MS/MS	20	144-2370	(Roberts and Thomas, 2006)
	River water	Germany	LC-MS/MS	12	60-152	(Hernando et al., 2006)
	Elbe River	Germany	GC-MS	0.05	8.7-32	(Weigel et al., 2004)
	Alster Lake	Germany	GC-MS	0.05	4.9	(Weigel et al., 2004)
	Alzette River	Luxembourg	LC-MS/MS	0.3	10-295	(Pailler et al., 2009)
	Mess River	Luxembourg	LC-MS/MS	0.3	9-2383	(Pailler et al., 2009)
	Surface water	South Korea	LC-MS/MS	1	11-38	(Kim et al., 2007a)
	Mankyung River	South Korea	LC-MS/MS	5	ND-414	(Kim et al., 2009)
	Surface water	UK	LC-MS/MS	20	ND	(Hilton and Thomas, 2003)
	Hoje River	Sweden	GC-MS	NA	90-250	(Bendz et al., 2005)
	Paraiba do Sul River	Brazil	GC-MS	10	ND-50	(Stumpf et al., 1999)
Naproxen	River water	Germany	LC-MS/MS	26	70	(Hernando et al., 2006)
	Pearl River	China	GC-MS	1.3	ND-118	(Zhao et al., 2009)
	Surface water	South Korea	LC-MS/MS	1	1.8-18	(Kim et al., 2007a)

Diclofenac was reported as high as 3600 ng/L in influent of wastewater treatment plant in Spain (Gomez et al., 2007). Similar concentrations were reported in Switzerland, Canada, and UK (Lee et al., 2005; Martinez Bueno et al., 2009; Roberts and Thomas, 2006; Tauxe-Wuersch et al., 2005). High concetrations of diclofenac was also reported in effluent in Spain (140-2200 ng/L and 890-1440 ng/L), Switzerland (1300-2400 ng/L), Canada (32-448 ng/L), UK (261-598 ng/L and 350-460 ng/L), and Belgium (32-1420 ng/L) (Gomez et al., 2006; Hernando et al., 2006; Hilton and Thomas, 2003; Martinez Bueno et al., 2009; Roberts and Thomas, 2006; Tauxe-Wuersch et al., 2005; Verenitch et al., 2006). There are also studies reporting low concentrations (8-250 ng/L) of diclofenac in influent and effluent (Bendz et al., 2005; Hernando et al., 2006; Kim et al., 2007a; Koutsouba et al., 2003; Pailler et al., 2009; Stumpf et al., 1999; Zhao et al., 2009).

Ibuprofen is the most studied NSAID and highest concentrations among pharmaceuticals were reported for ibuprofen. For instance, 34000-168000 ng/L and 240-28000 ng/L of ibuprofen were measured in Spain in influent and effluent, respectively (Gomez et al., 2007). UK is another country that ibuprofen concentrations were observed at extremely high concentrations in influent (7741-33764 ng/L) and effluent (1979-2370 ng/L) of wastewater treatment plants (Roberts and Thomas, 2006). High concentrations were also observed in Switzerland (1750-400 ng/L in influent and 100-1200 ng/L in effluent), Canada (4100-10210 ng/L in influent and 2235-6718 ng/L in effluent), Romania (110-2170 ng/L in effluent), Belgium (18-1860 ng/L in effluent), Taiwan (711-17933 ng/L in influent and 313-3777 ng/L in effluent), and UK (1700-3800 ng/L in effluent) (Hernando et al., 2006; Lin and Tsai, 2009; Lin et al., 2009; Moldovan, 2006; Tauxe-Wuersch et al., 2005; Verenitch et al., 2006).

Naproxen is the least studied compound among NSAIDs. Concentrations of naproxen in wastewater and treated wastewater are similar to concentrations of diclofenac. Naproxen concentrations was reported as 1730-6030 ng/L in influent and 360-2540 ng/L in effluent in Canada (Lee et al., 2005), 271-7962 ng/L in effluent in another study in Canada (Verenitch et al., 2006), 3650 ng/L in influent and 250 ng/L in effluent in Sweden (Bendz et al., 2005), 109-455 ng/L in effluent in Spain (Hernando et al., 2006), 31 ng/L in effluent in USA (Thomas and Foster, 2004), 625 ng/L in effluent in Belgium (Hernando et al., 2006), 38-320 ng/L in influent and 12-

139 ng/L in effluent in Japan (Nakada et al., 2006), 20-483 ng/L in effluent in South Korea (Kim et al., 2007a).

Concentration differences of pharmaceuticals and hormones among the countries and even in the countries indicate that it is not possible to predict concentrations of pharmaceuticals and hormones in regional basis.

2.3 Fate and Behavior of Pharmaceuticals and Hormones in Aquatic Environment

Residues of various pharmaceuticals are present in the low µg/L range in wastewater treatment plant effluents. Discharge of the wastewater treatment plan effluent into receiving waters leads to a dilution of the pharmaceutical residues which occur up to the high ng/L range in contaminated surface water. Once introduced into the surface waters, pharmaceuticals may undergo biodegradation, most likely due to co-metabolic processes. For some pharmaceuticals, i.e. diclofenac, photo induced degradation may occur from natural solar radiation (Andreozzi et al., 2003). Additionally, depending on the lipophilicity and specific sorption properties of a particular pharmaceutical, distribution between aqueous solution and sediment and suspended matter occurs (Schwarzenbach et al., 2003). Sorption to particular matter, or formation of bound residues might result in a change in the transformation behavior. However, the extent of pharmaceutical sorption to particulate matter is hardly known. Therefore, further research is still needed on the fate and behavior of pharmaceuticals and hormones in the aquatic environment (Santos et al., 2010). After use or disposal of pharmaceuticals and hormones they are introduced to the environment mainly through wastewater treatment plants or from agricultural lands via runoff. Since most of the pharmaceuticals and hormones are resistant to degradation they reach to surface water and eventually groundwater (Daughton and Ternes, 1999; Halling-Sorensen et al., 1998; Heberer, 2002). However, there are some findings indicating they may undergo some degradation processes such as photolysis which strongly depends on intensity of solar irradiation, latitude, season of the year and presence of photosensitizes (e.g. nitrates, humic acids) (Bartels and von Tuempling, 2007; Boreen et al., 2003; Santos et al., 2010).

Since pharmaceuticals and hormones are highly polar and not volatile, they are easily transported in the aquatic environment and even through food chain (Crane et al.,

2006; Daughton and Ternes, 1999). In developed countries, wastewater treatment plants are considered main sources of pharmaceuticals and hormones. However, there are some regions even countries this is not valid since wastewater collection and treatment cannot be established scientifically. Moreover, wastewater treatment plants' removal efficiency of pharmaceuticals and hormones may be dramatically different from plant to plant (Roberts and Thomas, 2006; Santos et al., 2010; Ternes, 1998).

Similar to other compounds of anthropogenic origin, the fate of the pharmaceuticals residues during sewage treatment can follow one or a combination of three types of behavior: a) (bio)degradation (mineralization), b) sorption of the residues onto sewage sludge or c) no elimination. The latter results in their presence in treated wastewater (Halling-Sorensen et al. , 1998).

Since pharmaceuticals and hormones have moderate to high log K_{oc} values, they either create organic complexes or be adsorbed to the sediment. After adsorption to the sediment, pharmaceuticals and hormones are become more available to be exposed to organisms, transformation, and degradation. If they are not adsorbed, they become more mobile and move in water column. Therefore, human and other organisms are open to both direct exposure and exposure via food web (Campbell et al., 2006).

The solubility values would suggest that most endocrine disrupters would generally not remain in solution. However, endocrine disrupters have been identified in water samples collected throughout the world (Ferguson et al., 2001; Petrovic et al., 2004; Rice et al., 2003; Thurman et al., 1992; Ying et al., 2002). In some cases endocrine disrupters have been found in groundwater and drinking water samples suggesting some type of soluble transport (López-Roldán et al., 2004; Petrovic et al., 2003). "Possible hypotheses for these observations include (1) more soluble precursors or metabolites, (2) colloid facilitated transport, (3) enhanced solubility through elevated pH (many endocrine disrupters have a pKa around 10), and (4) the formation of micelles. The formation of micelles can greatly enhance the stability of a compound, as well as facilitate the stability of other low solubility endocrine disrupters in solution" (Campbell et al., 2006).

2.3.1 Effects of pharmaceuticals and hormones to aquatic organisms

Adverse effects of chemicals to living organisms were determined with information obtained from toxicity experiments. Toxicity of a chemical depends on concentration and exposure time. During toxicology tests living organisms are used. Therefore, toxicology tests are also called bioassays. To obtain reproducible results, toxicity tests are conducted under standardized conditions. These conditions are designed to establish that only variable is tested chemical. After standardized tests, data can be presented as "concentration-response" or "dose-response" curves after proper statistical treatment. These curves represent degrees of responses to definite concentration or dose of the chemical (Rand, 1995). Bioassays, particularly chronic toxicity tests, are essential tools for risk analysts to predict possible environmental hazards (Ostrander, 1996).

There are different types of toxicity tests. It is possible to divide the toxicity tests into two main groups as *in vivo* tests and *in vitro* tests. *In vivo* tests are conducted using whole organisms to find out acute, chronic, and sub-chronic effects of chemicals. *In vivo* tests are conducted using isolated cell systems to find out genotoxicity or cell transformation (Hodgson, 2004).

In order to extrapolate meaningful, relevant, and ecologically significant results from aquatic toxicity tests appropriate organisms should be used. Several criteria that should be considered in selecting organisms for toxicity testing are proposed by Rand (1995):

1. Because sensitivities vary among species, a group of species representing a broad range of sensitivities should be used whenever possible.
2. Widely available and abundant species should be considered.
3. Whenever possible, species should be studied that are indigenous to or representative of the ecosystem that may receive the impact.
4. Recreationally, commercially, or ecologically important species should be selected.
5. Species should be amenable to routine maintenance in the laboratory and techniques should be available for culturing and rearing them in the laboratory so as to facilitate both acute and chronic tests.

6. If there is adequate background information on a species (i.e. physiology, genetics and behavior), the data from a test may be more easily interpreted.

Although mass measurements are necessary for fate and transport studies of pharmaceuticals and hormones, they do not provide information on ecological effects (*e.g.,* ecotoxicological, endocrine disruptive) of these chemicals (Campbell et al., 2006).

Endocrine disruption is another adverse effect of chemicals to living organisms. Detection of estrogenicity occurs by a number of mechanisms, including cell proliferation, ligand binding, vitellogenin induction, luciferase induction, or antigen–antibody interaction. These tests can be divided into three groups as whole organism assays, cellular bioassays, and non-cellular assays (Campbell et al., 2006).

Measuring endocrine disruption using whole organism assays relies on observation of change in population dynamics, reproduction deficiencies, gonad development, and vitellogenin synthesis in higher organisms such as amphibians, avian, and fish. Cellular estrogenicity bioassays can be summarized as YES, ER-CALUX, and E-SCREN. The most used non-cellular estrogenicity bioassays are the enzyme-linked immunosorbent assays (ELISA) and the enzyme-linked receptor assay (ELRA). Among estrogenicity bioassays ER-CALUX has the lowest detection limit with 0.14 ng/L. E-SCREEN and YES tests come after ER-CALUX with 0.27 ng/L and 0.3 ng/L detection limits, respectively (Campbell et al., 2006).

Pharmaceuticals are principally designed to persist in the body after administration. That might be the reason that many pharmaceuticals such as the lipid regulator clofibric acid, the antiepileptic carbamazepine or the contrast medium diatrizoate are relatively resistant towards degradation under environmental conditions and pass through the STP without major elimination (Ternes, 1998; Ternes and Hirsch, 2000).

Approximately 70% of the ecotoxicological studies of pharmaceuticals and hormones in the literature are on acute toxicity of them. Only 30 % of those studies deal with chronic effects. Growth inhibition, reproduction, immobilization, survival are the most used endpoints (Santos et al., 2010).

Since antibiotics are designed to cure diseases via adversely affecting organisms, they are intersection of environmental contamination and human health protection. The main problem caused by antibiotics is development of antibiotic resistance of

microorganisms and consequently losing effectiveness of antibiotics (Crane et al., 2006; Sanderson et al., 2004). They also have deleterious effects to higher organisms (Table 2.5).

Table 2.5: Literature survey on ecotoxicological effects of studied antibiotics.

Compound	Species	Toxicological Endpoint	Ecotoxicity Data	Reference
Amoxicillin	*M. aeruginosa*	EC50 (72h growth inhibition)	3.7 µg/L	(Lutzhoft et al., 1999)
	P. subcapitata	NOEC (72h growth inhibition	250 mg/L	(Lutzhoft et al., 1999)
	S. leopoliensis	EC50 (growth inhibition)	2.2 µg/L	(Andreozzi et al., 2004)
	S. leopoliensis	NOEC (growth inhibition)	0.78 µg/L	(Andreozzi et al., 2004)
	V. fischeri	EC50 (15 min, inhibition in luminescence)	3597 mg/L	(Park and Choi, 2008)
Erythromycin	*D. magna*	EC50 (24h immobilization)	22.45 mg/L	(Isidori et al., 2005b)
	C. dubia	EC50 (24h immobilization)	10.23 mg/L	(Isidori et al., 2005b)
	C. dubia	EC50 (7d population growth inhibition)	0.22 mg/L	(Isidori et al., 2005b)
	P. subcapitata	EC50 (72h growth inhibition)	0.02 mg/L	(Isidori et al., 2005b)
	P. subcapitata	EC50 (72h growth inhibition)	0.037 mg/L	(Eguchi et al., 2004)
	P. subcapitata	NOEC (72h growth inhibition)	0.01 mg/L	(Eguchi et al., 2004)
Sulfamethoxazole	*D. magna*	EC50 (48h immobilization)	189 mg/L	(Kim et al., 2007b)
	V. fischeri	EC50 (15 min, inhibition in luminescence)	78 mg/L	(Kim et al., 2007b)
	D. magna	EC50 (24h immobilization)	25.2 mg/L	(Isidori et al., 2005b)
	C. dubia	EC50 (24h immobilization)	15.5 mg/L	(Isidori et al., 2005b)
	C. dubia	EC50 (7d population growth inhibition)	0.21 mg/L	(Isidori et al., 2005b)
	P. subcapitata	EC50 (72h growth inhibition)	0.52 mg/L	(Isidori et al., 2005b)
	P. subcapitata	EC50 (72h growth inhibition)	1.53 mg/L	(Eguchi et al., 2004)
	D. magna	EC50 (48h immobilization)	123 mg/L	(Park and Choi, 2008)

Erythromycin was found to be the most ecotoxic antibiotic in the literature. Different species responded differently to antibiotics. Crustaceans (*D. magna* and *C. dubia*) are more resistant to ecotoxicological effects of antibiotics than algae (*P.*

subcapitata). Among crustaceans *C. dubia* is more sensitive. While acute effects of antibiotics were at mg/L levels, chronic effects ware observed at μg/L levels.

The main property of β-blockers is inhibition of β-receptors which are responsible for sympathetic responses (*e.g.,* heart rate increase) in vertebrates. While propranolol inhibits both of the β1 and β2 receptors, atenolol inhibits only β1 receptors (Santos et al., 2010). Although invertebrates such as algae do not possess β receptors they have been affected by β-blockers. Moreover, vertebrates' chronic exposure of β-blockers may cause drastic effects such as heart and liver failure (Table 2.6).

Table 2.6: Literature survey on ecotoxicological effects of studied β-blockers.

Compound	Species	Toxicological Endpoint	Ecotoxicity Data	Reference
Atenolol	*D. magna*	EC50 (48h immobilization)	313 mg/L	(Cleuvers, 2005)
	P. promelas	NOEC (28d growth inhibition)	3.2 mg/L	(Winter et al., 2008)
	P. promelas	NOEC (21d reproduction)	10 mg/L	(Winter et al., 2008)
Propranolol	*D. magna*	EC50 (48h immobilization)	7.5 mg/L	(Cleuvers, 2003)
	D. magna	LC50 (48h mortality)	1.6 mg/L	(Huggett et al., 2002)
	H. azteca	LC50 (48h mortality)	29.8 mg/L	(Huggett et al., 2002)
	H. azteca	NOEC (24d reproduction)	1 μg/L	(Huggett et al., 2002)
	C. dubia	LC50 (48h mortality)	0.8 mg/L	(Huggett et al., 2002)
	C. dubia	NOEC (7d reproduction)	0.125 mg/L	(Huggett et al., 2002)
	D. magna	NOEC (9d body mass)	0.22 mg/L	(Dzialowski et al., 2006)

Atenolol and propranolol have very different effects to organisms even though they are member of same therapeutic group. It may be due to propranolol's blocking of both β-receptors while atenolol blocks only one β-receptor.

Estrogens are mostly reported hormones existing in environmental waters. It is known that they have vitellogenin synthesis, vitelline envelope (eggshell) protein production, gonadal differentiation, development of secondary sexual characteristics, GnRH and gonadotropin secretion, oestrogen receptor synthesis, pheromonal

communication, bone formation and calcium homeostasis effects to fish (Larsson et al., 1999). Vitellogenin concentrations can be found in the blood plasma of male fish when they had been exposed to estrogens. High concentrations of vitellogenin in the blood plasma of male fish causes feminization or simultaneous occurrence of male and female gonadal characteristics (Jobling et al., 1998).

Oral contraceptive pills contain synthetic estrogen, EE2 which has highest endocrine disruptive effect among estrogen hormones (Larsson et al., 1999). Chronic exposure of fathead minnows to EE2 at concentrations lower than 1 ng/L causes higher egg production but lower fertilization. Concentrations over 3.5 ng/L of EE2 causes totally feminization of all of the male fish (Parrott and Blunt, 2005; Santos et al., 2010) (Table 2.7).

Table 2.7: Literature survey on ecotoxicological effects of studied hormones.

Compound	Species	Toxicological Endpoint	Ecotoxicity Data	Reference
E2	*O. latipes*	NOEC (21d testis-ova induction)	<29.3 ng/L	(Kang et al., 2002)
EE2	*P. promelas*	LOEC (21d plasma VTG induction)	1 ng/L	(Pawlowski et al., 2004)
	D. rerio	LOEC (38d plasma VTG induction)	2 ng/L	(Orn et al., 2003)

Estrogenic responses of hormones were detected using ER-CALUX and YES tests. It is reported that EE2 had 1.2 estradiol equivalent estrogenicity in both of the tests. While 0.1 estradiol equivalent estrogenicity was found in YES test, it was 0.056 estradiol equivalent in ER-CALUX for E1 (Murk et al., 2002).

NSAIDs are responsible to inhibit cyclooxygenase enzymes, COX-1 and COX-2. Since fish have a cyclooxygenase enzymes resembling human COX-2 enzyme, they might be affected directly or via food web by NSAIDs (Santos et al., 2010).

Diclofenac, ibuprofen, and naproxen have different effects on aquatic organisms (Table 2.8).

Table 2.8: Literature survey on ecotoxicological effects of studied hormones.

Compound	Species	Toxicological Endpoint	Ecotoxicity Data	Reference
Diclofenac	*D. magna*	EC50 (48h immobilization)	72 mg/L	(Cleuvers, 2003)
	D. magna	EC50 (48h immobilization)	68 mg/L	(Cleuvers, 2004)
	D. magna	EC50 (48h immobilization)	22 mg/L	(Cleuvers, 2003)
	D. magna	EC50 (48h immobilization)	108 mg/L	(Cleuvers, 2003)
	P. subcapitata	NOEC (96h growth inhibition)	10 mg/L	(Ferrari et al., 2003)
	D. subcapitatus	EC50 (growth inhibition)	72 mg/L	(Cleuvers, 2003)
	C. dubia	EC50 (48h immobilization)	22 mg/L	(Ferrari et al., 2003)
	C. dubia	NOEC (7d reproduction)	1 mg/L	(Ferrari et al., 2003)
	D. rerio	NOEC (10d survival)	4 mg/L	(Ferrari et al., 2003)
Ibuprofen	*D. magna*	EC50 (48h immobilization)	108 mg/L	(Cleuvers, 2003)
	D. magna	EC50 (48h immobilization)	10-100 mg/L	(Heckmann et al., 2007)
	D. magna	EC50 (14d reproduction)	13.4 mg/L	(Heckmann et al., 2007)
	D. subcapitatus	EC50 (growth inhibition)	315 mg/L	(Cleuvers, 2003)
	D. subcapitatus	EC50 (growth inhibition)	342 mg/L	(Cleuvers, 2004)
	O. lapites	LC50 (96h mortality)	>100 mg/L	(Pounds et al., 2008)
Naproxen	*D. magna*	EC50 (48h immobilization)	174 mg/L	(Cleuvers, 2003)
	D. magna	EC50 (48h immobilization)	166 mg/L	(Heckmann et al., 2007)
	C. dubia	EC50 (24h immobilization)	66 mg/L	(Isidori et al., 2005a)
	C. dubia	NOEC (7d reproduction)	0.33 mg/L	(Ferrari et al., 2003)
	P. subcapitata	NOEC (72h growth inhibition)	32 mg/L	(Isidori et al., 2005a)
	D. subcapitatus	EC50 (growth inhibition)	626 mg/L	(Cleuvers, 2004)

There are few studies in the literature on mixture effects of pharmaceuticals and hormones. Some of them indicate mixture of pharmaceuticals may exert additive effects (DeLorenzo and Fleming, 2008). On the other hand, (Cleuvers, 2003)

showed synergistic interaction between diclofenac and ibuprofen during *D. magna* immobilization test. Moreover, *D. magna* immobilization was observed for acetylsalicylic acid, diclofenac, ibuprofen, and naproxen mixture even all of them are at concentrations which they do not affect *D. magna* when they are single (Cleuvers, 2004). These studies are very limited and do not provide general information on interactive effects of pharmaceuticals and hormones.

3. MATERIALS AND METHOD

Experimental approach of this study consisted of two main elements which are field study and laboratory study (Figure 3.1).

3.1 Sampling

3.1.1 Description of the watershed

İstanbul with its population over 10 million and average rate of population increase of 4.9% is one of the greatest metropolitan cities in the world (Maktav and Erbek, 2005). Due to climate change and global warming as well as the huge amount of migration it receives, local authorities and central government face a challenge with supplying drinking water to the residents of Istanbul and meeting the required demands of drinking water quality. For instance, between 1998 and 2007, the amount of water supplied per year increased from 598,742,000 m^3 to 732,051,000 m^3.

Over 90% of the water demand of İstanbul is supplied from surface water, currently from six drinking water reservoirs. Of the six watersheds that supply drinking water, three are located on the European side (Terkos, Büyükçekmece, and Alibeyköy) and three on the Asian side (Ömerli, Darlık, and Elmalı) of the city (Figure 3.2). Moreover, there are minor drinking water resources such as Istırancalar, Sazlıdere, Pabuçdere, and Kazandere creeks.

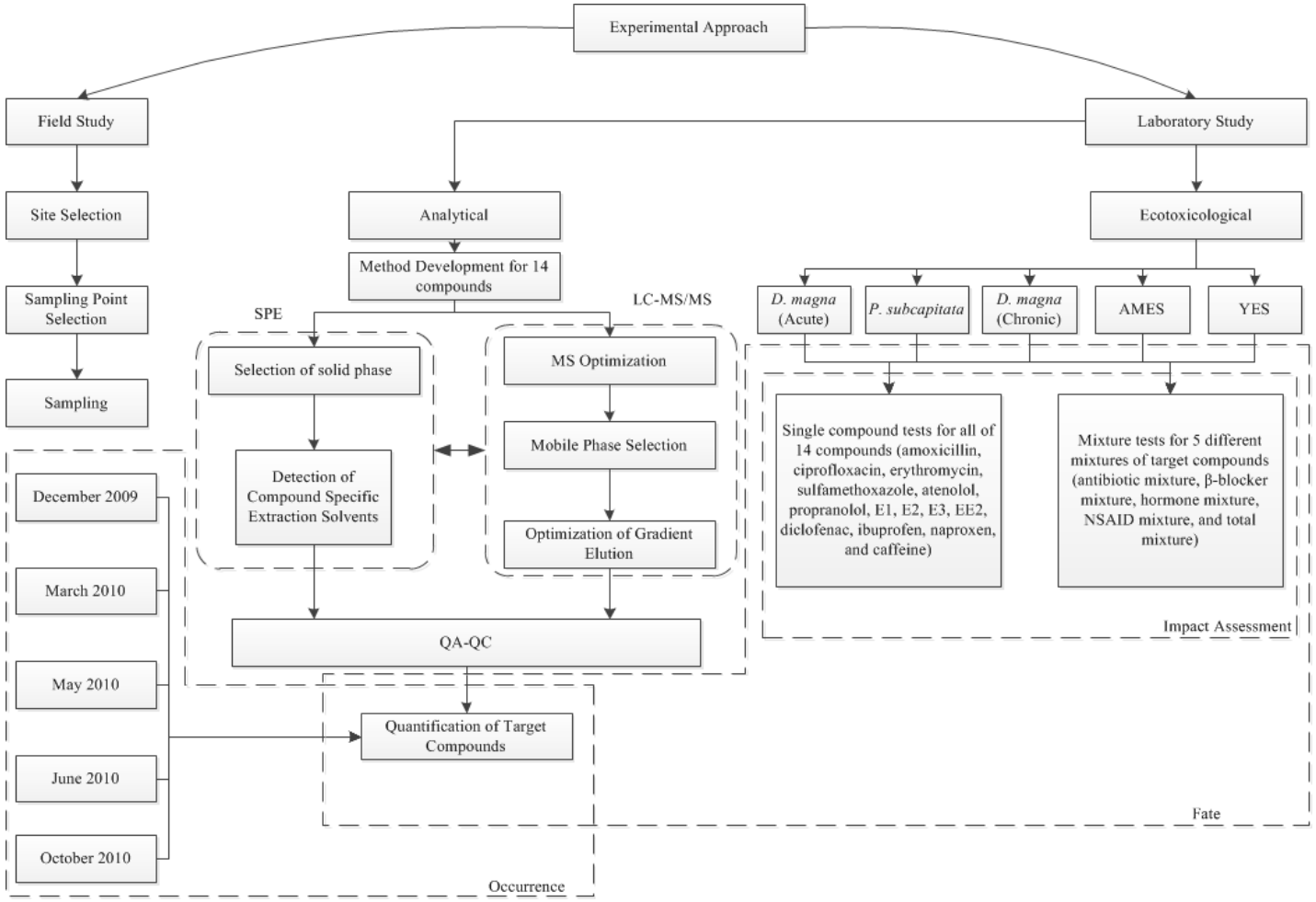


Figure 3.1: Experimental approach of the study.

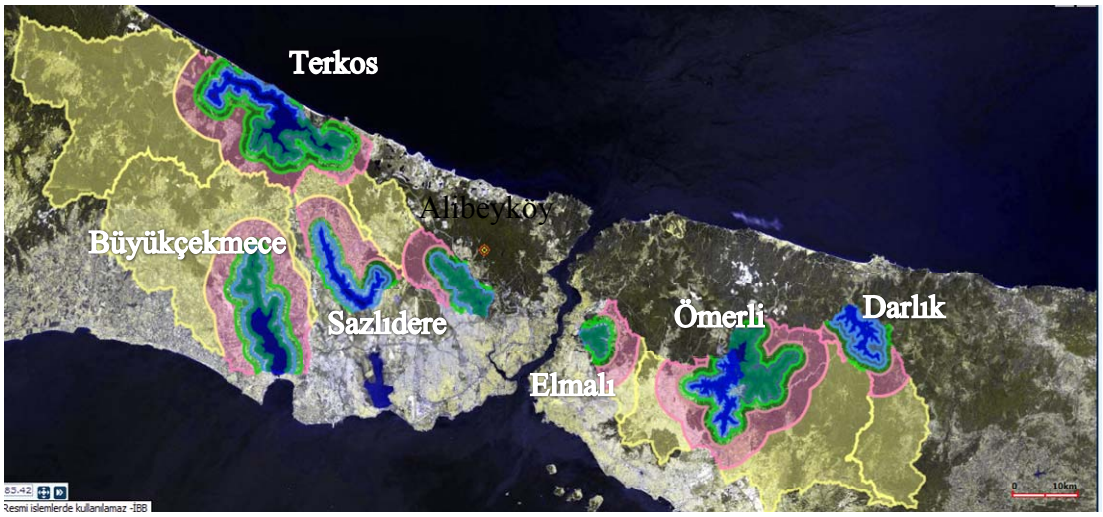


Figure 3.2: Drinking water watersheds of Istanbul.

Büyükçekmece Lake, covering 27.5 km^2 area and lying in 620 km^2 watershed, is the third important water source for İstanbul making the lake and its watershed very important for inhabitants of İstanbul (Table 3-1).

Table 3.1: Extractable water amounts of drinking water resources of Istanbul in 2008.

Reservoir	Extractable Water in May 2008 (million m^3)
Ömerli	91
Terkos	130
Büyükçekmece	70
Darlık	32
Alibeyköy	6
Elmalı	6
Sazlıdere	16
Istırancalar	1
Kazandere	0.1
Pabuçdere	0.7

After 2008, water extracted from Melen River started to be used in Istanbul. The annual amount of water brought from Melen depends on rain rate and changed between 2 million m^3 and 134 million m^3 from 2008 to 2012.

Büyükçekmece Watershed is one of the important migration taking areas in Istanbul. Today, approximately 180,000 inhabitants live in the watershed and its projected population for 2020 is 260,000 (Baykal et al., 2000). Most of the inhabitants live in the long range protection zone (i.e. area corresponding to 2000 m to watershed boundary from the lake). However, there are some small communities living in the absolute protection zone due to unplanned urbanization although it is banned by regulations.

The most recent study on land use of the watershed was 2000 indicating 12% of the area was residential and industrial (Maktav and Erbek, 2005). However, considering the population increase, increase in these areas is most likely.

There are five main tributaries flowing into Büyükçekmece Lake: Beylikçayı, Karasu, Hamza, Tahtakoprü, and Ahlat (Figure 3.3). Karasu is the greatest one with 70 km approximate length and 275 km^2 sub-watershed. Karasu passes through Çatalca which is the greatest town in the watershed and found as having 4th degree water quality regarding N and P in Water Pollution Control Regulation (Gönenç, 1995).

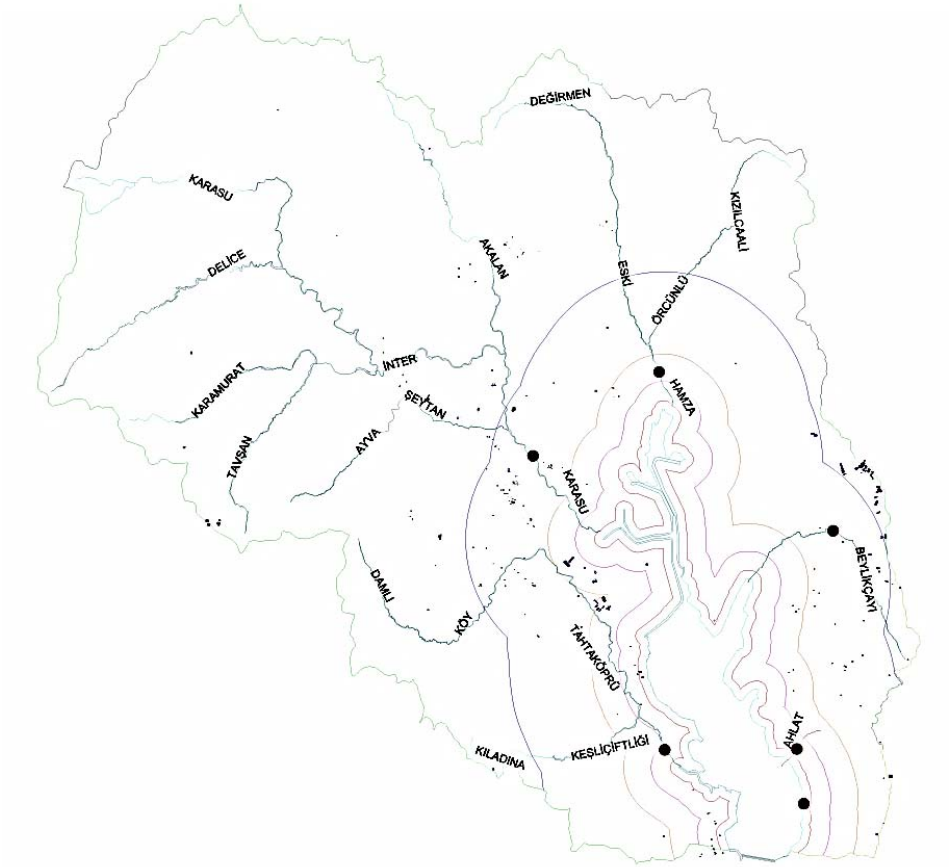


Figure 3.3: Büyükçekmece Watershed.

Büyükçekmece Lake was designated as polluted surface water in Istanbul City Environmental Situation Report. Residents, industries, erosion, and agriculture were considered as main pollution sources (İÇDR, 2007). In addition, Büyükçekmece Lake was found 3rd class regarding organic parameters, 4th class regarding inorganic parameters, and 2nd class regarding biological parameters according to the classification in Water Pollution Control Regulation (Baykal et al., 2000).

According to the data obtained by Istanbul Water and Sewerage Administration in 2006, there are 287 industries in the watershed. Most of the industries are in food, metal, chemistry, textile, leather, and petroleum industrial categories. Moreover, only 30% of them have wastewater treatment plant. Nevertheless, while 16 of the industries are placed in the lake absolute protection zone, 60 of them are in river absolute protection zone.

Büyükçekmece Water Treatment Plant lies at the southeast of the lake. Capacity of the treatment plant is 400,000 m^3/day. The quality of the treated water has been routinely checked by Istanbul Water and Sewerage Administration and published monthly reports. It is stated in these reports that treated water of the water treatment plant meets all drinking water standards. However, no emerging pollutants in particular pharmaceuticals and hormones are included in these standards. Therefore, pharmaceuticals and hormones are not monitored in untreated and treated water of the watershed. Consequently, there is no information on environmental and human health risks posed by pharmaceuticals and hormones in this area.

3.1.2 Sampling sites

Grab samples were taken from six different stations, five on each main tributary and one on the lake (Figure 3.4).

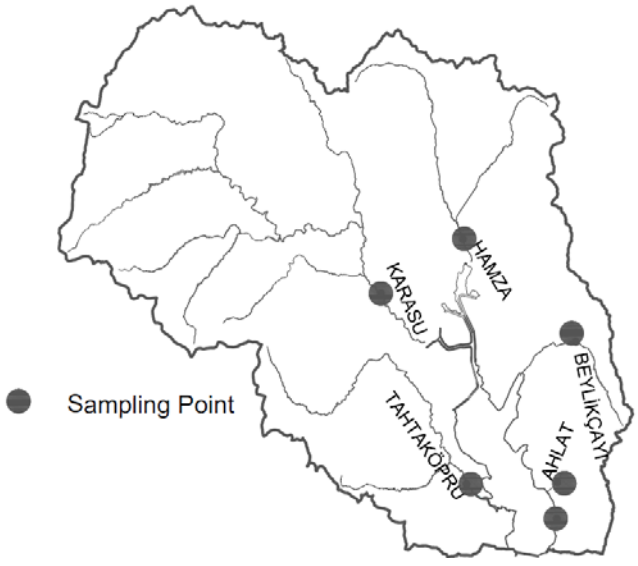


Figure 3.4: Sampling points.

Sampling points on the tributaries were determined as close as possible to the lake to be able to monitor all pollution loads flowing into the lake. Sampling point on the lake was selected as close as possible to water intake structure of the water treatment

plant. Sampling was made five different times in a year (December, March, May, July, October) to examine seasonal changes.

3.2 Compound selection

Pharmaceuticals to be monitored should be selected according to their consumptions, excretion rates and types. There were around 21,000 pharmacies in Turkey in 2003. In the pharmaceutical sector, there are 87 manufacturing firms, 11 raw material manufacturers, and 38 importing firms, summing up 136 firms. The top ten bestselling pharmaceutical preparations account for 40% of the total market. Antibiotics, analgesics, and antirheumatic preparations are the most sold pharmaceuticals in 2005 in terms of boxes of drugs sold (Kisa, 2006). However, "boxes of drugs sold" is not a proper unit for estimating the drug use, since it does not provide information on the "mass" of active ingredient of the drug.

The pharmaceutical usage rates between October 2005 and October 2007 in Days of Therapy (DOT) unit is provided in Table 3.2 along with World Health Organization (WHO) Anatomical Therapeutic Chemical (ATC) classification codes.

DOT is a measure of pharmaceutical use that indicates direct measure of the number of days of therapy. One DOT represents the administration of a single agent on a given day regardless of the number of doses administrated or dosage strength. Polk et al. (2007) indicated that DOT methodology is a superior measure of use and can be used to compare relative uses of different pharmaceuticals (Polk et al., 2007).

Table 3.2: Pharmaceutical usage rates in DOTs.

Therapeutic Class	DOT
M01 ANTIRHEUMATIC SYSTEM	1,124,269,015
A02 A-ACID A-FLAT A-ULCERANTS	894,334,215
J01 SYSTEMIC ANTIBACTERIALS	873,980,787
B03 ANTIANAEMICS	767,845,588
A10 DRUGS USED IN DIABETES	765,560,971
R01 NASAL DECONG/ANTIINFECT.	748,137,864
H03 THYROID THERAPY	716,004,419
C10 LIPID-REG/ANTI-ATHEROMA	638,142,697
N06 PSYCHOANALEPTICS	629,244,137
C09 RENIN-ANGIOTEN SYST AGENT	625,492,084
C08 CALCIUM ANTAGONISTS	445,432,491
R05 COUGH & COLD PREPARATIONS	440,934,939
G03 SEX HORMONES-SYSTEMC ONLY	424,310,288
R03 ANTI-ASTHMA & COPD PROD	348,142,596
A11 VITAMINS	322,928,483
R06 ANTIHISTAMINES SYSTEMIC	294,112,997
C01 CARDIAC THERAPY	259,822,230
N02 ANALGESICS	237,636,959
C07 BETA BLOCKING AGENTS	220,779,878
G04 UROLOGICALS	168,353,661
N05 PSYCHOLEPTICS	156,584,388
C03 DIURETICS	146,425,081
A03 FUNCTL.GI DISORDER DRUG	122,843,749
N03 ANTI-EPILEPTICS	116,776,786
B01 ANTITHROMBOTIC AGENTS	108,322,682

Source: IMS Health Turkey (personal communication)

Pharmaceuticals to be monitored were selected according to usage rates, excretion rates and types and hence the probable importance in the environment. a total of ten pharmaceuticals including three NSAIDs, Diclofenac, Ibuprofen, Naproxen; four antibiotics amoxicillin, ciprofloxacin, erythromycin, and sulfamethoxazole; two β-blockers, atenolol and propranolol; and one stimulant, caffeine were selected. Estrogen hormones estrone (E1), estradiol (E2), estriol (E3), and 17α-ethynylestradiol (EE2) were also selected to observe their occurrence and fate in aquatic environment. All of the compounds have different chemical and physical properties (Table 3.3 and Table 3.4). Therefore, it is likely that they will have different behaviors in the environment.

Table 3.3: Selected compounds and their main properties.

Compound	CAS No	Use	Molecular Weight (g/mol)	Log K_{ow}	Excretion rate as unchanged compound (%)
Amoxicillin	267-87-78-0	Antibiotic	365.4	0.97	80-90[2]
Ciprofloxacin	85721-33-1	Antibiotic	331.3	0.28	83.7[2]
Erythromycin	114-07-8	Antibiotic	734	3.06	15[2]
Sulfamethoxazole	723-46-6	Antibiotic	253.3	0.89	15[2]
Atenolol	29122-68-7	β-Blocker	266.3	0.16	90[3]
Propranolol	525-66-6	β-Blocker	259.3	3.48	<1[4]
Estrone (E1)	53-16-7	Hormone (Natural)	270.4	3.13	3-20[5]
17β-Estradiol (E2)	50-28-2	Hormone (Natural)	272.4	4.01	0.5-5[5]
Estriol (E3)	50-27-1	Hormone (Natural)	288.4	2.45	<64[5]
17α-Ethynylestradiol (EE2)	57-63-6	Hormone (Synthetic)	296.4	3.67	40[6]
Diclofenac	15307-86-5	NSAID[1]	318.1	4.51	15[2]
Ibuprofen	15687-27-1	NSAID[1]	206.3	3.97	1-8[2]
Naproxen	22204-53-1	NSAID[1]	230.3	3.18	2[7]
Caffeine	58-08-2	Stimulant	194	-0.07	0.4-2.1[8]

[1]NSAID: Non-steroidal anti-inflammatory drug
[2](Jjemba, 2006)
[3](Zuccato et al., 2005)
[4](Ternes and Joss, 2006)
[5] typical daily excretion amount in µg/d, (Birkett and Lester, 2003)
[6](Johnson and Williams, 2004)
[7](Bougie and Aster, 2001)
[8](Birkett and Miners, 1991)

Table 3.4: Molecular structures of target compounds.

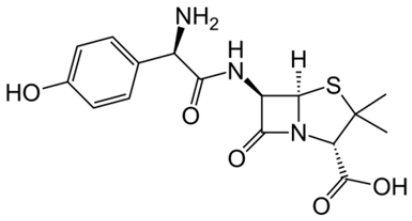
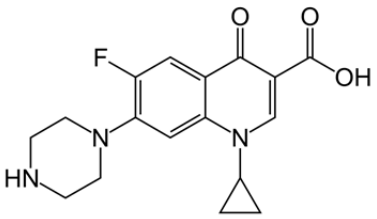
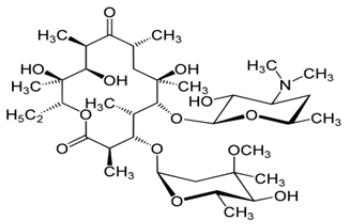
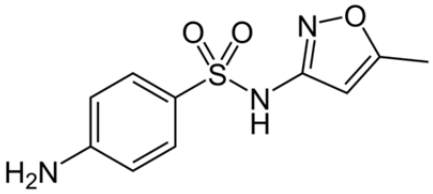
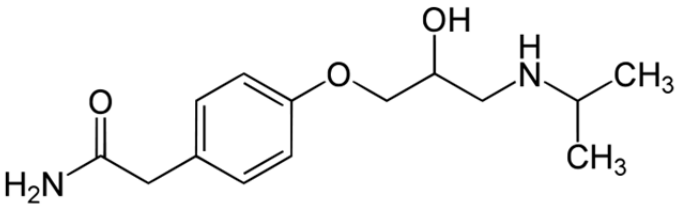
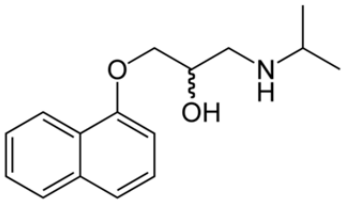
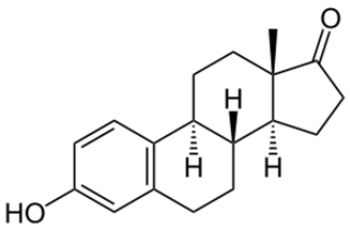
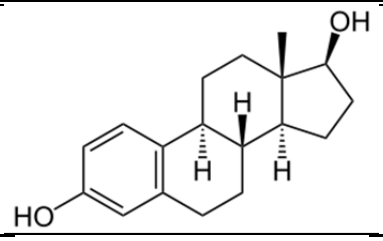
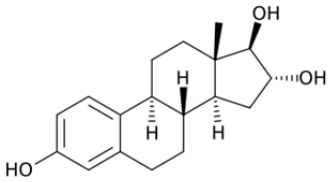
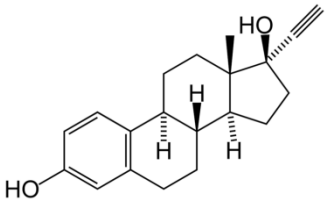
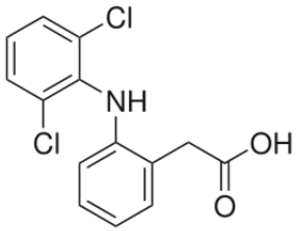
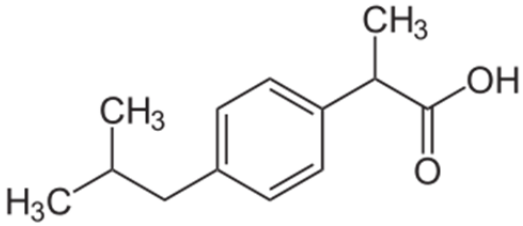
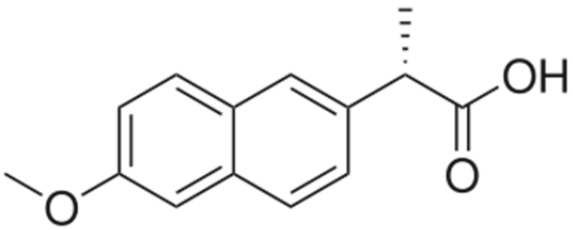
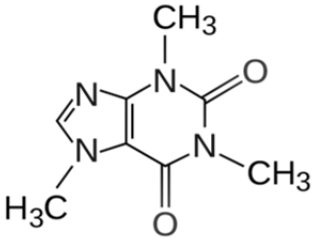
Compound	Molecular structure
Amoxicillin	
Ciprofloxacin	
Erythromycin	
Sulfamethoxazole	
Atenolol	
Propranolol	
E1	

Table 3.4 (continued): Molecular structures of target compounds.

E2	
E3	
EE2	
Diclofenac	
Ibuprofen	
Naproxen	
Caffeine	

3.3 Analytical Method

3.3.1 Standards and reagents

Target compounds were purchased from Sigma-Aldrich. All of them were of analytical grade, purity 95% or higher. Among isotopically-labelled internal/surrogate standards, d_2-Estradiol was purchased from Sigma-Aldrich, $^{13}C_2$-17α-Ethynylestradiol, d_2-Ibuprofen, d_7-Atenolol, d_8-Ciprofloaxacin•HCl were purchased from C/D/N Isotopes. Glass Oasis HLB cartridges (200 mg, 5 mL) were purchased from Waters Corporation and used for solid phase extraction (SPE). HPLC-gradient grade methanol, acetonitrile, acetone and methyl *tert*-butyl ether (MTBE) were obtained from Sigma-Aldrich as well as LC-MS grade formic acid. 25% NH_4OH was supplied from Merck. High purity water (conductivity less than 0.056 μS/cm^3) was obtained from Sartorius Stedim Digitech Arium 611 UV model distilled water generator. Nitrogen gas for analyte enrichment (99.995%) and argon (99.999%) were purchased from Linde Gas. Nitrogen gas for nebulizing and desolvation (high purity) was provided by a nitrogen generator of Peak Scientific Instrument NM 30LA 230VOC.

All stock standards were prepared in acetonitrile and stored in +4°C for three months except for antibiotics which were renewed monthly and stored in dark in amber bottles to avoid photodegradation. Working solutions were prepared in water using stock solutions before each measurement.

3.3.2 Sample preparation

Samples were taken with Nalgene fluorinated jerricans. All samples were filtered through 0.22 μm Whatman Polycap AS 75 filters within 24 hours after sampling. Oasis HLB cartridges are used for SPE of multi-residue analysis of pharmaceuticals in different therapeutic classes (Gracia-Lor et al., 2011; Gros et al., 2006; 2009). The SPE cartridges were conditioned with 5 mL methyl tert-butyl ether, 5 mL acetone, 5 mL methanol, 5 mL acetonitrile and 5 mL deionized water. One liter filtered sample was spiked with 40 μL of 2.5 mg/L surrogate/internal standard solution and then loaded on to SPE cartridges at 3-5 mL/min. After sample loading, the cartridges are washed with 5 mL deionized water and dried under vacuum for 60 minutes. The SPE cartridges were eluted with 5 mL methyl tert-butyl ether, 5 mL acetone, 5 mL methanol and 5 mL acetonitrile. The extracts then were evaporated until dryness

under gentle stream of nitrogen (0.5 bar) using Caliper TurboVap II system. The analytes were reconstituted using 1 mL of 20:80 acetonitrile:water mixture. In addition, prior to loading the samples onto SPE cartridges, 1 g Na_2EDTA was added to improve the extraction efficiency of antibiotics Gros et al. (2009).

3.3.3 LC-MS/MS analysis

LC-MS/MS analysis was conducted using a Thermo Electron Cooperation Accela UPLC coupled with TSQ Quantum Access triple quadrupole mass spectrometer with electronspray ionization (ESI). A Thermo Hypersil Gold column (100 mm x2.1 mm i.d., 1.9 μm,) was used. Although there may not be necessary chromatographic separation of target compounds during tandem mass spectrometric analysis, gradient elution was developed in order to prevent cross talks in MS. Three mobile phase lines of UPLC were used for both negative ionization (NI) and positive ionization (PI) modes. In each mode, one line was dedicated to the buffer solution and the percentage of this line was kept constant during the entire run. Consequently, buffer was added to organic solvents and buffer capacity was kept stable during the whole run. In the PI mode, mobile phase A, B, and C were 1% formic acid, acetonitrile and ultra-pure water, respectively. In the NI mode, mobile phase A, B, and C were 50 mM NH_4OH, acetonitrile and ultra-pure water, respectively (Table 3.5). 400 μL/min flowrate and 25 μL injection volume were used in all runs. While column temperature was set to 25°C, autosampler tray was kept at 10°C.

Table 3.5: Gradient elution programs of LC.

PI Mode				NI Mode			
Time (min)	A (%)	B (%)	C (%)	Time (min)	A (%)	B (%)	C (%)
0	10	10	80	0	20	15	65
8	10	90	0	4.5	20	80	0
8.7	10	90	0	5	20	80	0
9	10	20	70	5.5	20	15	65
12	10	20	70	8	20	15	65

Compound dependent MS parameters (spray voltage (SV), sheath gas pressure (SGP), auxiliary gas pressure (AGP), ion sweep gas pressure (ISGP), capillary temperature (CT), tube lens offset (TLO), collision energy (CE), and collision pressure (CP)) and two transition ions were detected via direct infusion of 500 μg/L of each compound at a flow rate of 10 μL/min using the syringe pump of the MS. In order to achieve

better sensitivity, different time segments were used which also lead to higher number of points per chromatographic peak. Common MS/MS parameters for the PI mode were SV: 5000 V; CT: 250°C; SGP: 30 arb; ISGP: 4 arb; AGP: 5 arb; ST: 50 ms; SW: 0.2 m/z; whereas they were determined as SV, -3500 V; SGP, 40 arb; ISGP, 2 arb; AGP, 20 arb; ST, 50 ms; SW, 0.01 m/z for the NI mode. Segment specific parameters, scan time intervals, SRM transitions and retention times were provided in Table 3.6.

Two transition ions were selected to use in SRM for each compound of interest except Ibuprofen which yields only one transition ion during triple quadrupole mass spectrometry due to poor fragmentation (Gros et al., 2009). The transition ion with the higher intensity was used for quantification (first transition in Table 3.6) and the other ion was used for confirmation (second transition in Table 3.6) to eliminate false positives (Schlusener and Bester, 2005). Only one transition ion was used for internal/surrogate standards, since they are not naturally found in environmental waters. d_2-Estradiol was used as surrogate/internal standard for quantification of E1 and E2, $^{13}C_2$-17α-Ethynylestradiol was used as surrogate/internal standard for quantification of EE2 and E3, d_2-Ibuprofen was used as surrogate/internal standard for quantification of Ibuprofen, Naproxen and Diclofenac, d_7-Atenolol was used as surrogate/internal standard for quantification of Atenolol, Propranolol and Caffeine, and d_8-Ciprofloaxacin was used as surrogate/internal standard for quantification of Sulfamethoxazole, Ciprofloxacin, Amoxicillin and Erythromycin.

To calculate recoveries during the SPE, ultra-pure water and one of the samples were spiked with different concentrations (10 ng/L and 100 ng/L, n=3 for each) of target compounds and each spiked sample were extracted using the proposed SPE procedure and analyzed. To eliminate the effect of the presence of target compounds in the sample prior to spiking non-spiked samples were also extracted and analyzed. Concentrations determined in non-spiked samples were subtracted from the concentrations of spiked samples during the calculation of the recovery

Table 3.6: Segment specific parameters, scan time intervals, SRM transitions, and retention times.

Compound	Time Segment (minute)	Retention Time (minute)	SRM transition	TLO	CE
Positive Ionization					
Amoxicillin	0-1.8	1.19	366.2=>114.11 366.2=>160.41	80	18
Ciprofloxacin	1.8-3.2	2.67	332.1=>287.83 332.1=>230.95	100	15
Erythromycin	3.2-7	4.89	716.5=>558.45 716.5=>157.95	68	20
Sulfamethoxazole	3.2-7	3.57	253.9=>155.92 253.9=>108.11	68	20
Atenolol	0-1.8	0.86	267.1=>190.07 267.1=>145.07	80	18
Propranolol	3.2-7	4.17	260=>155.07 260=>183.07	68	20
Caffeine	1.8-3.2	2.12	195=>138 195=>110	100	15
d7-Atenolol	0-1.8	0.86	274.1=>191.90	80	18
d8-Ciprofloaxacin	1.8-3.2	2.67	340.1=>296.15	100	15
Negative Ionization					
Diclofenac	0-2.5	2.14	294=>249.90 294=>214.02	50	12
Ibuprofen	0-2.5	1.86	205.4=>161.4	50	12
Naproxen	0-2.5	1.02	229.3=>170.1 229.3=>169.1	50	12
d2-Ibuprofen	0-2.5	1.86	208.2=>164.2	50	12
E1	2.5-6	4.75	269=>145.07 269=>143.24	105	40
E2	2.5-6	4.48	271.1=>182.96 271.1=>145.12	105	40
E3	2.5-6	3.11	287=>170.87 287=>145	105	40
EE2	2.5-6	4.68	295=>145.1 295=>185.1	105	40
d2-E2	2.5-6	4.48	173=>147.2	105	40
$^{13}C_2$-EE2	2.5-6	4.68	297=>159	105	40

3.4 Ecotoxicological Experiments

The effects of the target compounds were determined using several ecotoxicological bioassays in this study. Experiments were designed to obtain information on lethal and sub-lethal effects of single compounds as well as on the possible effect when the compounds coexist as a mixture. Moreover, since the determination of

ecotoxicological effects of chemicals to a single species do not provide enough information, four different species are used to determine four different effects (*i.e.* acute, chronic, mutagenic, and estrogenic) of target compounds. While acute effects were determined using *P. subcapitata* (freshwater algae growth inhibition test) and *D. magna* (immobilization test), only *D. magna* is used for the determination of chronic effects (reproduction test). Mutagenicity was determined using the AMES test (mutant *S. tphidyum*). YES test (recombinant *S. cerevisiae*) was used for determination of estrogenic effects.

All ecotoxicological experiments were conducted using synthetic solutions of target compounds and all solutions were prepared in water media proper for the test conducted.

3.4.1 Acute toxicity tests

Pseudokirchneriella subcapitata and *Daphnia magna* were used in acute toxicity tests. Both of the species are ecologically important. *P. subcapitata* are unicellular freshwater green algae. They are primary producers like all other green algae species. Therefore, any adverse effects to them threaten the whole ecosystem. *P. subcapitata* are one of the recommended species by OECD in its standard for ecotoxicity tests. Since they are commonly used for ecotoxicity assays, they are commercially available. *D. magna* are freshwater crustacean. They occupy an important part of the food web. They are predators of primary producers and prey of carnivore aquatic animals. Therefore, any adverse effects on them may pose threat to both primary production process and carnivores. *D. magna* are also a commonly used species for ecotoxicity tests and are commercially available. Both of the species have different sensitivities to different chemicals. Although *P. subcapitata* are considered more sensitive than *D. magna*, it is not valid for all of the chemicals. However, their sensitivities are good enough to be used in ecotoxicological studies.

3.4.1.1 *Daphnia magna* acute immobilization test

Acute immobilization tests of water flea *D. magna* were conducted according to the OECD 202 standard (OECD, 2004). 24-hour and 48-hour exposure times were used as recommended in the standard method. *D. magna* populations were incubated under standard conditions to establish that the only variable is the test material. First brood of the population was not used as recommended in the standard. *D. magna*

incubation media consists of four stock solutions: 11.76g $CaCl_2 \cdot 2H_2O$ was dissolved in 1 liter distilled water, 4.93 g $MgSO_4 \cdot 7H_2O$ was dissolved in 1 liter distilled water, 2.59g $NaHCO_3$ was dissolved in 1 liter distilled water, 0.23g KCl was dissolved in 1 liter distilled water. 25 mL of each solution were mixed and made up to 1L with distilled water and oxygenated to prepare media. This media has a hardness of 140-250 mg $CaCO_3$/L, a pH of 7.8, a Ca/Mg molar ratio of approximately 4, and a dissolved oxygen concentration above 7. Other important variables in the test are the light and temperature. During incubation, the population kept in a 16-hour light (800 lux intensity) and 8-hour dark cycle. On the other hand, tests were conducted in dark. All incubations and tests were conducted in a constant temperature room having a temperature of 20±2°C. Populations were fed with *P. subcapitata* and yeast during incubation.

Members of the population younger than 24 hours were exposed to different concentrations of compounds in four replicates in vessels designated for this test. In each replicate 5 individuals were used. All solutions of the test compounds as well as dilutions were prepared in the media of *D. magna*. A dilution-water control was also conducted for each test.

All test results were examined using SigmaPlot statistical program and different end points (EC10, EC50, EC80 and if possible NOEC and LOEC) were estimated via plotting the appropriate curve using appropriate non-linear regression method (*e.g.* probit, weibull).

3.4.1.2 Freshwater algae growth inhibition test

Although growth inhibition tests of freshwater algae were using *P. subcapitata* is considered as an acute toxicity test, it is called as semi-chronic or chronic toxicity test in some test protocols and standards depending on the test duration. The test duration, or the exposure time, may be the main difference between acute and chronic tests, but it is not the only factor. In chronic tests, covering important part of life span of test organisms and conducting tests in semi-static or continuous are essential to observe chronic effects. Since freshwater algae tests were conducted static and in 72-hour exposure time, it is called as acute toxicity test in this study.

P. subcapitata populations were grown in a media recommended in the standard (Table 3.7).

Table 3.7: Algae growth medium stock solutions.

Nutrients	Concentration in stock solution (mg/L)
Stock solution 1: macro nutrients	
NH_4Cl	1500
$MgCl_2 \cdot 6H_2O$	1200
$CaCl_2 \cdot 2H_2O$	1800
$MgSO_4 \cdot 7H_2O$	1500
KH_2PO_4	160
Stock solution 2: iron	
$FeCl_3 \cdot 6H_2O$	64
$Na_2EDTA \cdot 2H_2O$	100
Stock solution 3: trace elements	
H_3BO_3	185
$MnCl_2 \cdot 4H_2O$	415
$ZnCl_2$	3
$CoCl_2 \cdot 6H_2O$	1.5
$CuCl_2 \cdot 2H_2O$	0.01
$Na_2MoO_4 \cdot 2H_2O$	7
Stock solution 4: bicarbonate	
$NaHCO_3$	50000

While stock solutions 1 and 3 were sterilized by autoclaving, 2 and 4 are filter-sterilized by membrane filters with a pore diameter 0.2 μm. To prepare th final growth medium 10 mL of the stock solution 1 and 1 mL of each of the stock solutions 2, 3, and 4 are added to 500 mL sterilized distilled water and finally made up to 1 L with sterilized distilled water. The prepared growth medium was left under open air in a laminar flow chamber for equilibration with atmospheric CO_2. All stock solutions were kept in amber glass bottles at 4°C. Solutions and dilutions of test substances were also prepared in the growth medium.

Incubation of *P. subcapitata* and tests were conducted in a temperature controlled room having a temperature 20±2°C. Constant/continuous light was provided with uniform daylight type florescent illumination. Light intensity was kept 6000 lux which is in the range of recommended light intensity (4440-8880 lux).

Algal biomass is used to compute growth and growth inhibition during a period of time. Dry weight of the algal population must be measured to find algal biomass. Since it is difficult to measure dry weight in particular this kind of bioassays due to very low weight, some other parameters such as cell counts are often used. In this study, cell counts were used as surrogate parameter to estimate growth inhibition. Cells were counted using a hemocytometer and an Olympus microscope (40x).

Exponentially growing test organisms were exposed to various dilutions of target compounds for 72-hour under certain conditions. Responses were evaluated in comparison with growth of exposed organisms and unexposed control cultures. All experiments were conducted with four replicates.

Each replicate of each dilution was inoculated with $1x10^4$ cells/mL (initial cell concentration). Inoculums used in the tests were prepared 2-4 days before the tests to let the population reach exponential growth phase and adapt alga to test conditions.

The system response is the reduction of growth in a series of algal cultures (test units) exposed to various concentrations of a test substance. The response is evaluated as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures. For full expression of the system response to toxic effects (optimal sensitivity), the cultures are allowed unrestricted exponential growth under nutrient sufficient conditions and continuous light for a sufficient period of time to measure reduction of the specific growth rate (OECD, 2006).

Specific growth rate was calculated as:

$$\mu_{i-j} = \frac{\ln X_j - ln\, X_i}{t_j - t_i} \quad \textbf{(3.1)}$$

where:

μ_{i-j} is specific growth rate between i and j

X_i is the biomass at time i

X_j is the biomass at time j

The percent inhibition of the growth rate was calculated as:

$$\%I_r = \frac{\mu_c - \mu_t}{\mu_c} x100 \quad \textbf{(3.2)}$$

where:

$\%I_r$: percent inhibition in average specific growth rate

μ_c: mean value for average specific growth rate in the control group

μ_t: average specific growth rate for the treatment replicate

3.4.2 *D. magna* reproduction test

This test was used to predict effects of chemicals on the reproductivity of *D. magna*. Less than 24-hour old female *D. magna* individuals were exposed to different concentrations of target compounds for 21 days. The total number of living offspring produced per parent animal alive at the end of the test was used to assess effects. The reproductivity of exposed animals was compared with the reproductivity of animals in the control groups to estimate the lowest observable effect concentrations (LOEC), no observable effect concentrations (NOEC) as well as the ECx values where available.

The same growth medium with the acute immobilization test described in 3.4.1.1 was used. The test solutions and dilutions were prepared in the same medium.

10 animals were maintained individually in 100 mL beakers containing 50 mL solutiion for each concentration. The tests were conducted in semi-static manner feeding all test animals daily with *P. subcapitata* and yeast as well as changing solutions three times in a week. To each animal, 0.1-0.2 mg C/day which is sufficient to achieve enough offspring to end the test was fed.

During incubation, the test animals kept in a 16-hour light (800 lux intensity) and 8-hour dark cycle. All incubations and tests were conducted in a constant temperature room having a temperature of 20±2°C.

During 21-day period, number of offspring in each beaker, number of dead parents, and any possible stress indicating anomalies such as losing color of the animals were noted (OECD, 1998).

3.4.3 AMES test

AMES test is the most widely used and accepted mutagenicity test based on bacterial reverse-mutation. The test employs a mutant strain, or several strains, of *Salmonella typhimurium*, carrying mutation(s) in the operon coding for the amino acid, histidine, biosynthesis. When these bacteria are exposed to mutagenic agents, reverse mutation from histidine auxotrophy to prototrophy occurs. Traditionally, reverse-mutation assays have been performed using agar plates, known as “pour plate”, “plate-incorporation” or “agar-overlay” assays (Ames et al., 1975). An alternate assay performed entirely in liquid culture is the `Fluctuation test', originally developed by

Luria and Delbruck (1943) and was modified by Kilbey (1984). In this study, The Muta-ChromoPlate^{TM} kit with TA100 mutant strains to perform the Fluctuation test was used.

All essential chemicals, growth media and test strains were provided with the kit. One day before the test lyophilized bacteria was reconstituted with nutrient broth supplied with the kit and incubated for 16-18 hours at 37°C. Reconstituted bacteria should have turbid yellowish color (Figure 3.5).

Figure 3.5: Reconstituted bacteria for AMES test.

On the test day samples were sterilized using membrane filters with 0.22 µm pore sizes. 17.5 mL of filtered samples were transferred to sterile falcon tubes. A reaction mixture consisting 21.62 mL concentrate Davis-Mingioli salts, 4.75 mL D-glucose, 2.38 mL Bromocresol Purple, 1.19 mL D-Biotin, 0.06 mL L-Histidine was prepared. 2.5 mL of reaction mixture were added to each sample, negative control, positive control and background. 5 µL of incubated and well mixed *S. typhimurium* test-strain broth culture were added to each treatment tube except negative control. Contents of the each tube were transferred to a sterile multichannel pipette reagent boat. 200 µL of the mixtures were dispensed into each well of a 96-well

microtitration plate using a multichannel pipette. At the beginning, color of each well must be purple. Well-plates were incubated for 5 days at 37°C.

In this test, negative control was used to determine whether there had been bacterial contamination in solutions. A well-known mutagen NaN_3 was used as positive control to control if the bacteria work. During replication of *S. typhimurium* natural reverse mutations may occur. To characterize how much natural reverse mutation occurs, background control was used. In background control, non-mutagen sterile distilled water was used as sample.

After 5 days all well-plates were observed. If reverse mutation had occurred, the bacteria had ability to synthesize histidine and consequently, caused color turned from purple to yellow.

Fluctuation test is based on comparison of number of the yellow wells in samples and number of yellow wells in background. If there is a statistically significant increase in the number of yellow wells in sample plate than the number of yellow wells in background plate, the sample is designated as mutagen.

3.4.4 YES test

A recombinant yeast strain, *Saccharomyces cerevisiae*, which can interact with the human estrogen receptor (hER) was used in Yeast Estrogen Screen Tests (YES). Normally, there is not any estrogen receptor in yeast cells. Therefore, the DNA sequence of hER should be stably added to their main chromosome. The receptors' activity is detected using expression plasmids carrying the reporter gene *lac-Z* (encoding the enzyme b-galactosidase) which is naturally contained in the yeast cells.

The biochemical reactions during the test were best explained in Jobling et al. (1996) and Isidori et al. (2006) as: In this system, the hER is expressed in a form capable of binding to estrogen-responsive sequences (ERE). These sequences were situated within a strong promoter sequence on the expression plasmid. Upon binding an active ligand, the estrogen-occupied receptor interacts with transcription factors and other transcriptional components to modulate gene transcription. This causes expression of the reporter gene *lac-Z* and the enzyme produced (b-galactosidase) is secreted into the medium, where it metabolizes the chromogenic substrate, *ortho*-nitrophenyl,β-D-galactopyranoside (ONPG), which is normally colorless, into a yellow product that can be measured by absorbance at 420 nm (Figure 3.6).

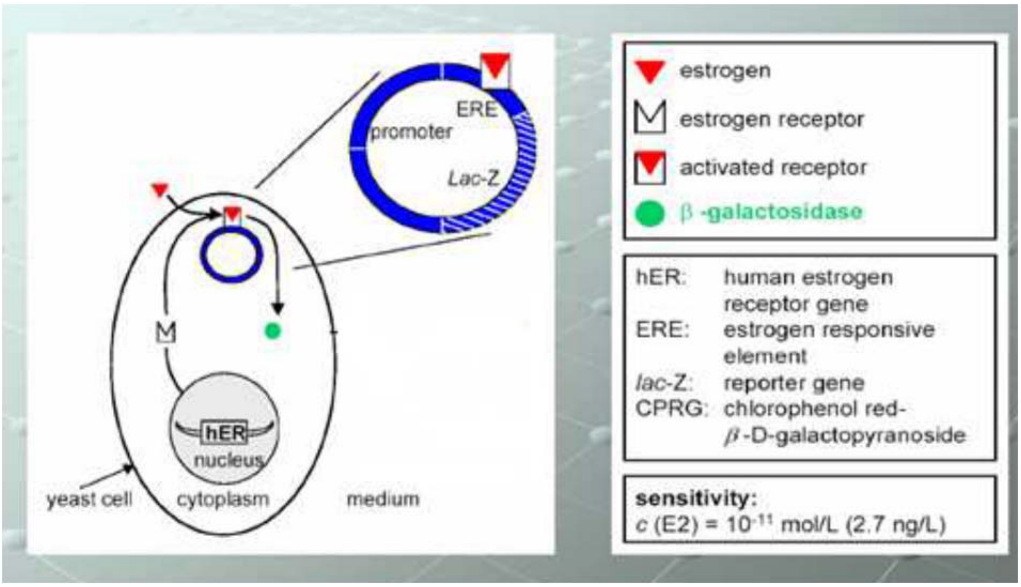


Figure 3.6: YES test main mechanism (Routledge and Sumpter, 1996).

S. cerevisiae RMY326 strain which was kindly supplied by Luigi Mita from Second University of Naples, Italy was used in this study.

The yeast cells, normally stored at -80°C, were reconstituted at 28°C with constant shaking at 200 rpm in a medium (Table 3.8) overnight.

Table 3.8: Yeast medium for YES test.

Substance	Concentration
Yeast Nitrogen Base	6.7 g/L
Glucose	2% (w/v)
Isoleucine (Ile)	30 mg/L
Valine (Val)	250 mg/L
Adenine (Ade)	50 mg/L
Arginine . HCl (Arg.HCl)	20 mg/L
Lysine . HCl (Lys.HCl)	30 mg/L
Methionine (Met)	20 mg/L
Phenylalanine (Phe)	50 mg/L
Threonine (Thr)	200 mg/L
Tyrosine (Tyr)	30 mg/L
Histidine . HCl (His.HCl)	200 mg/L
Leucine (Leu)	100 mg/L

After 16-18 hours the yeasts reached exponential growth phase. The yeasts having concentration of $2x10^7$ cells/mL were incubated in the presence of the target compounds for another 16-18 hours at 28°C. Duplicates of five different concentrations were used for each compound as well as each mixture to obtain data for statistical evaluation of endpoints. Along with target compounds a blank and the duplicates five different concentrations of E2 ($1x10^{-5}$ – $1x10^{-9}$ M) were added to each

test as positive control. Consequently, interferences caused by any contaminations and daily fluctuations on standard (E2) values were avoided.

After second incubation, 1 mL of the samples as well as blank and standards were centrifuged at 4000 rpm for 5 minutes and the supernatant was discarded. The yeast cells were re-suspended in 150 µL z-buffer (30 mM Na_2HPO_4, 20 mM NaH_2PO_4, 5mM KCl, 0.5 mM $MgSO_4$, 0.025% (v/v) b-mercaptoethanol). 50 µL of re-suspensions were taken to small vials and 50 µL CH_2Cl_2, 20 µL SDS and 30 µL z-buffer were added for permeabilization of cell membranes. The vials were vortexed for 10 seconds and incubated at 28°C for 5 minutes. 700 µL ONPG (4mg/mL in z-buffer) were added to each vial for chromogenic reaction and all vials were incubated at 28° C. After approximately 5 minutes, chromogenic reaction was stopped by the addition of 500 µL of 1 M Na_2CO_3. All vials were centrifuged at 14000 rpm for 5 minutes and the absorbance at 420 nm was measured. In the final step, absorbance of 1 mL of non-centrifuged samples after second incubation at 600 nm was measured to determine cell density in the incubation tubes. The results of the tests were presented as Miller Unit (MU) calculated as:

$$MU = \frac{OD_{420} x 1000}{txVxOD_{600}} \quad \textbf{(3.3)}$$

where:

MU: Miller Unit

OD_{420}: absorbance at 420 nm

OD_{600}: absorbance at 600 nm

t: chromogenic reaction time

V: Volume of the culture used in the test (50 µL in this case)

The Relative Inductive Efficiency (RIE) which is the estrogenic activity of the tested compound relative to E2 was determined as the ratio of the maximal β-galactosidase activity induction with test compound to E2×100.

YES test was not conducted for hormones since their estrogenic effect is natural.

3.5 Statistical Analysis

All data obtained in the experiments were treated with proper statistical analysis. Analytical measurements during the occurrence study were conducted in duplicates and samples were injected triplicates to be able to calculate standard deviations. All experiments and injections were made in triplicates to determine the detection limits of analytical methods.

Acute toxicity, chronic toxicity, and YES tests were conducted in four, ten, two replicates, respectively. For the AMES Test, a statistical approach designed in particular for this test, fluctuation test, was used (Luria and Delbruck, 1943).

Linearization of plots with logarithmic scale used to be the most commonly used technique for calculation of ECx values. However, non-linear regression analysis has recently become more popular to treat ecotoxicological data due to enhanced robustness of the non-linear regression and the development of computerized tools for curve fitting. Therefore, non-linear regression was used with the help of a computer program (*i.e.* SigmaPlot) in this study. Appropriate non-linear regression method (*e.g.* probit, weibull, sigmoidal) was selected according to the fitting of the curves to ecotoxicology data. The LOEC and hence the NOEC were estimated using the ANOVA analysis.

4. RESULTS AND DISCUSSIONS

4.1 Analytical Measurement Method Development

4.1.1 Solid phase extraction

Since Oasis HLB cartridge consists of a hydrophilic-lipophilic balanced adsorbent, it is quite capable of adsorbing compounds having different polarities. However, elution of adsorbed compounds seems to be problematic. Different solvents having different polarities were used in SPE procedure in order to tackle this problem.

To efficiently elute less polar compounds such as hormones solvents less polar than acetonitrile and methanol was used. Although dichloromethane with methanol did not provide good recoveries, MTBE with methanol was essential to achieve high recoveries for hormones. On the other hand, these two solvents were not enough to efficiently elute other compounds in particular antibiotics. Therefore, acetonitrile and acetone were added to SPE elution step.

All recoveries were between 60 and 119 % (Table 4.1). While caffeine and naproxen had excellent recoveries, antibiotics, and E1 had fair recoveries. Generally, recoveries in ultra-pure water were better than recoveries in river water. This is due to matrix effect which is the main drawback of ESI.

Drying time of elution may also affect recoveries. Since none of the target compounds are volatile, evaporation until dryness and hence long evaporation times (~1.5 hour) does not cause any adverse effect.

Table 4.1: Recoveries of the compounds.

Compound	Recovery, % (RSD, %)			
	Ultra-pure water		River water	
	10 ng/L	100 ng/L	10 ng/L	100 ng/L
β-Blockers				
Atenolol	94 (7.3)	98 (6.7)	80 (11.2)	85 (10.7)
Propranolol	88 (10.3)	91 (9.6)	71 (14.9)	77 (13.5)
Antibiotics				
Amoxicillin	72 (12.5)	77 (12.7)	61 (13.5)	67 (13.4)
Ciprofloxacin	87 (9.3)	104 (6.7)	64 (10.2)	75 (9.9)
Erythromycin	73 (9.2)	79 (9.2)	61 (13.4)	66 (12.6)
Sulfamthoxazole	85 (10.2)	92 (9.8)	75 (11.9)	78 (11.2)
NSAIDs				
Diclofenac	88 (9.3)	93 (8.8)	72 (12.2)	86 (11.4)
Ibuprofen	97 (7.3)	99 (6.9)	67 (11.1)	73 (10.3)
Naproxen	98 (7.5)	99 (7.2)	93 (8.3)	95 (8.1)
Hormones				
E1	81 (9.4)	86 (9.1)	63 (14.3)	76 (13.1)
E2	96 (9.1)	105 (8.5)	71 (13.2)	85 (11.9)
E3	96 (8.9)	119 (8.1)	81 (10.7)	93 (10.1)
EE2	92 (9.6)	94 (9.2)	84 (10.6)	90 (9.9)
Stimulant				
Caffeine	99 (7.1)	99 (6.5)	96 (8.4)	98 (7.9)

4.1.2 LC-MS/MS analysis

Acetonitrile was selected as the organic solvent in this method, because higher sensitivities were achieved for hormones with acetonitrile rather than methanol.

Some studies indicate that NH_4OH enhances ionization during NI mode detection (Kasprzyk-Hordern et al., 2008a; Yamamoto et al., 2006). The enhancement of signal intensity depends on the concentration of mobile phase additive. While low concentrations of mobile phase additive may not be enough to enhance the signal intensity, high concentrations cause decreases in signal intensity. Different NH_4OH concentrations were evaluated in order to determine the optimum NH_4OH concentration and it was determined as 10 mM (Figure 4.1). Previous studies suggest addition of either formic or acetic acids to promote positive ionization of compounds. In this study, formic acid which provided good chromatographic separation, sensitivity, and peak shape was used (Figure 4.2).

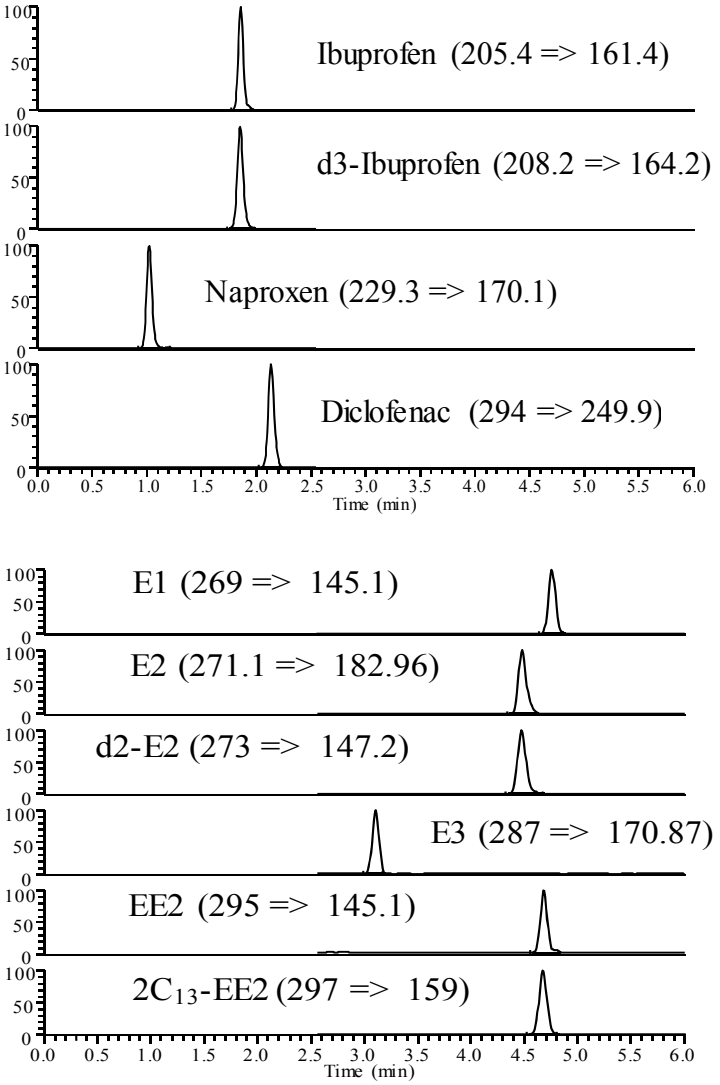


Figure 4.1: An example of chromatograms in negative ionization mode.

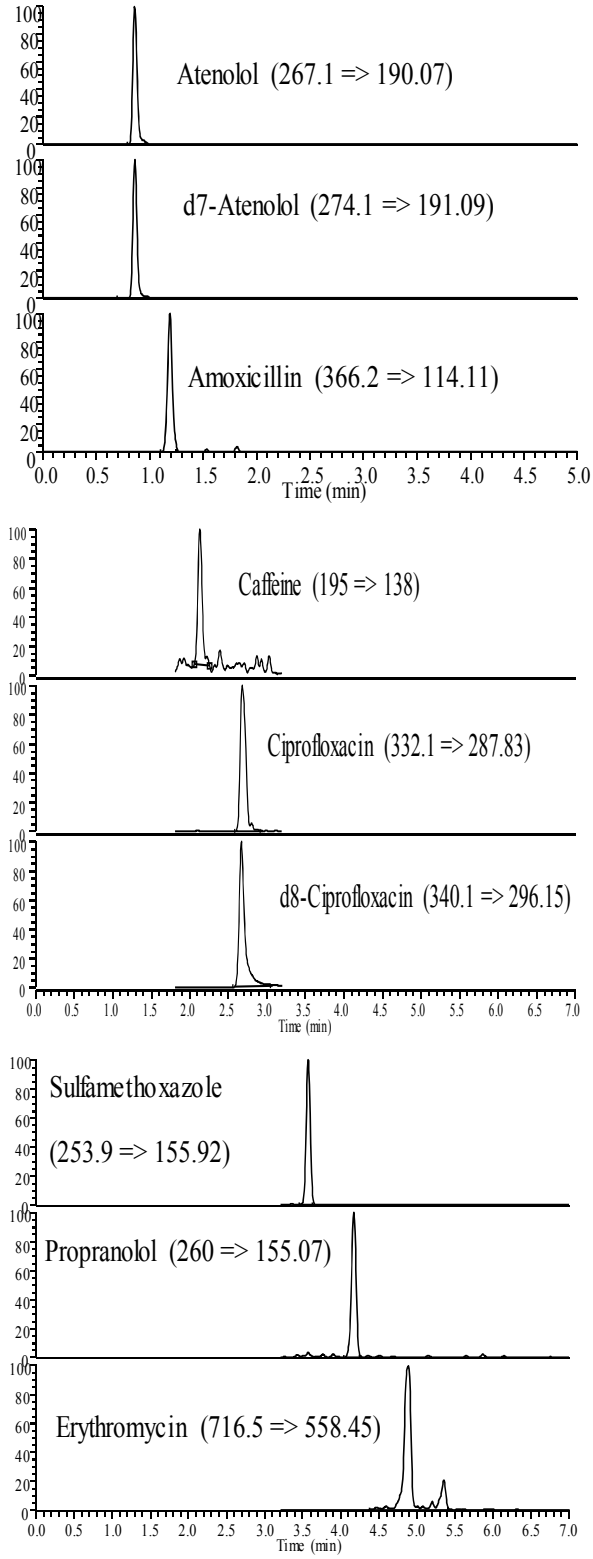


Figure 4.2: An example of chromatograms in positive ionization mode.

Since changing the pH of solvent is not recommended, additives are generally used only in water. This causes a change in the buffer capacity of the mobile phase during gradient elution. A substance leaving column near the end of a run may not be

ionized well enough during reversed phase chromatography. To prevent this problem, three lines of LC system were used. One mobile phase line was dedicated to ultra-pure water with additive and the percentage of this solution was not changed during the whole run. Based on the results of preliminary trials, sensitivity, particularly for the compounds leaving column towards the end of the run were higher than in two-line system.

Protonated and deprotonated ions were used for all MS/MS transitions of PI and NI modes, respectively. Intensity of ionization varied among compounds due to existence of different functional groups in molecular structures. Caffeine, naproxen, and diclofenac had highest intensities. Although the lowest intensities among the compounds were achieved for the hormones, the MQLs were still as low as 1 ng/L for all of the hormones.

Two peaks were observed for erythromycin which is parallel to other studies in literature (Vanderford et al., 2003). The highest peak was used for quantification (Figure 4.2).

4.1.3 Quality assurance/Quality control

In order to determine signal suppression due to sample matrix, areas of the peaks of the compounds in spiked ultra-pure water and spiked samples were compared. The highest signal suppression, more than 60%, was observed for diclofenac, naproxen and E3. Signal suppressions for erythromycin, sulfamethoxazole, E1, and caffeine were fairly low (<20%). To eliminate quantification errors due to signal suppression methods such as sample extract dilution (Gros et al., 2006), standard addition and internal standard calibration can be used. Among these methods, calibration with internal standard that is chemically similar to the analyte or the isotopically labelled form of the analyte is the most commonly used technique for quantification of pharmaceuticals and hormones in environmental waters, because it is less time consuming than other methods. Since isotopically labelled standards are not commercially available for all compounds and existing ones are very expensive, it is not possible to use internal standards for all compounds. As a matter of fact, the lack of compound-specific internal standards is the main limitation for analysis of pharmaceuticals and hormones in environmental matrices (Gros et al., 2009;

Kasprzyk-Hordern et al., 2008b). The suitability of internal/surrogate standards was evaluated whether they can prevent quantification errors due to ion suppression.

Method detection limit (MDL) and method quantification limit (MQL) were estimated from sample injections where signal-to-noise ratios of 3 and 10, respectively. Since MDL and MQL were slightly different in different sample matrices, averages were calculated in order to report one figure for each. Instrument detection limit (IDL) was determined via injection of series of dilutions of standards until to a signal-to-noise ratio of 3. For the compounds tested, IDL, MDL and MQL were in the range of 0.1-12.25 pg, 0.1-0.5 ng/L and 0.5-1.3 ng/L, respectively. While highest sensitivities were achieved for atenolol and diclofenac (MQL=0.5 ng/L) the method had lowest sensitivity for propranolol (MQL=1.3ng/L). Results of SPE recoveries, IDL, MDL, MQL and signal suppressions are provided in Table 4.2.

Table 4.2: Instrumental/method detection limits, method quantification limit and signal suppression.

Compound	IDL (pg)	MDL (ng/L)	MQL (ng/L)	Signal suppression (%)
β-Blockers				
Atenolol	2.5	0.25	0.5	31
Propranolol	6.25	0.5	1.3	39
Antibiotics				
Amoxicillin	12.5	1	1.5	32
Ciprofloxacin	1	0.1	1	23
Erythromycin	0.25	0.2	0.7	17
Sulfamethoxazole	0.625	0.1	1	16
NSAIDs				
Diclofenac	0.125	0.1	0.5	67
Ibuprofen	1.25	0.2	1.1	54
Naproxen	0.1	0.2	0.9	63
Hormones				
E1	0.625	0.5	1	17
E2	1.5	0.5	1	41
E3	2.5	0.5	1	67
EE2	2.5	0.5	1	54
Stimulant				
Caffeine	6.25	0.5	1	15

50 µg/L of a standard mixture were injected 5 times per day in different days in order to calculate repeatability and reproducibility. Relative standard deviations (RSDs) of repeatability and reproducibility tests were lower than 9% and 17%, respectively.

Calibration curves were estimated as linear curves using 1/x weighing least square regression. Each calibration curve had at least 0.99 R^2 value. Concentration range of calibration curves were 1-100 µg/L which yields 1-100 ng/L concentration rage after a concentration factor of 1000 by SPE. 7 point-internal standard calibration was used for quantification. Standards were injected three times in each run scattered throughout whole run to prevent errors caused by possible fluctuations.

4.2 Occurrence of the Pharmaceuticals and Hormones

Measurement results of samples taken in February are provided in Table 4.3.

Table 4.3: Concentrations of pharmaceuticals and hormones in February.

	Lake	Karasu	Tahtakoprü	Hamza	Ahlat	Beylikçayı
Antibiotics						
Amoxicillin	BQL	10.10	4.80	BQL	48.10	9.20
Ciprofloxacin	11.50	BDL	4.40	4.50	44.50	BDL
Erythromycin	0.70	1.60	1.00	0.90	7.90	BQL
Sulfamethoxazole	BQL	6.30	4.50	2.60	9.90	4.30
β-Blockers						
Atenolol	4.70	2.40	4.00	1.20	20.20	BDL
Propranolol	BDL	BDL	BDL	BDL	BDL	BDL
Hormones						
E1	BDL	1.10	BQL	BQL	BDL	BQL
E2	1.10	BDL	BDL	BDL	BDL	1.10
E3	4.60	3.10	3.70	1.90	4.00	3.40
EE2	BDL	BDL	BDL	BDL	BDL	BDL
NSAIDs						
Diclofenac	1.70	1.20	5.30	8.30	8.10	1.80
Ibuprofen	29.10	BDL	BDL	14.20	BDL	108
Naproxen	8.30	75.20	88.60	129	2.60	411
Stimulant						
Caffeine	32.60	1290	46.80	21.40	46.70	5525

All concentrations are in ng/L
BDL: Below Detection Limit
BQL: Below Quantification Limit

Caffeine concentrations over µg/L level in Karasu and Beylikçayı indicate wastewater contamination in these tributaries. The most polluted tributary was Beylikçayı in this sampling term with high caffeine, ibuprofen, and naproxen concentrations. Another factor caused these high concentrations in Beylikçayı was low flow rate respect to other tributaries. Another small tributary, Ahlat, had highest antibiotic concentrations among all sampling points in this sampling period. On the other hand the greatest river in the watershed, Karasu, had relatively high naproxen

and caffeine concentrations meaning loads of these compounds were also high. Concentrations in the lake were generally lower than its tributaries. Still, relatively high concentrations were observed for ciprofloxacin, ibuprofen, E3, and caffeine. Main source of amoxicillin and ciprofloxacin was Ahlat. Both of the antibiotics flow into the lake nearly the same concentration from Ahlat. Moreover, amoxicillin was measured 10.10 ng/L and 9.20 ng/L in Karasu and Beylikçayı, respectively, but ciprofloxacin was not detected in those rivers. It can be considered that volume of the lake is that higher to neglect volumes of the tributaries flowing into the lake. Therefore, it would have been expected that concentration of amoxicillin in the lake would be higher than concentration of ciprofloxacin. However, while ciprofloxacin was measured 11.50 ng/L amoxicillin concentration was below quantification limit in the lake. These results indicate that amoxicillin is prone to sink processes in the environment and ciprofloxacin more resistant to natural removal than amoxicillin.

Measurement results of samples taken in March are provided in Table 4.4.

Table 4.4: Concentrations of pharmaceuticals and hormones in March.

	Lake	Karasu	Tahtakoprü	Hamza	Ahlat
Antibiotics					
Amoxicillin	4	21.4	7.9	9.1	40.6
Ciprofloxacin	6.7	14.8	7.1	6.6	32.6
Erythromycin	BDL	7.1	9.7	0.9	4.2
Sulfamethoxazole	1.5	63.9	11.2	23	15.8
β-Blockers					
Atenolol	0.7	17.1	6.9	3.7	33.4
Propranolol	BDL	BDL	BDL	BDL	BDL
Hormones					
E1	BQL	BDL	BDL	BDL	1.4
E2	1.1	1.3	BDL	1	2
E3	2.9	3.2	1.3	1.2	6.2
EE2	11.6	BDL	BDL	BDL	BDL
NSAIDs					
Diclofenac	5.5	7.8	2.9	1.2	BDL
Ibuprofen	BDL	35	50.3	13.8	112
Naproxen	26.1	501	134	82.2	484
Stimulant					
Caffeine	1228	50.9	953	1107	20426

All concentrations are in ng/L
BDL: Below Detection Limit
BQL: Below Quantification Limit

Sample cannot be taken from Beylikçayı in March. The most interesting result in this term is approximately 20 µg/L concentration of caffeine in Ahlat. This high

concentration indicates wastewater domination in Ahlat in this sampling term. While approximately 1 μg/L caffeine concentration was measured in the Lake, Tahtakoprü, and Ahlat, 51 ng/L caffeine was measured in Karasu which was in contrast to the results of samples taken in February. High caffeine concentration in the Lake indicates not only concentrations but also loads of caffeine were high. Ahlat was the most polluted tributary in this sampling term with highest concentrations for all of the compounds except erythromycin, EE2, and naproxen.

Measurement results of samples taken in May are provided in Table 4.5.

Table 4.5: Concentrations of pharmaceuticals and hormones in May.

	Lake	Karasu	Tahtakoprü	Hamza	Ahlat	Beylikçayı
Antibiotics						
Amoxicillin	BQL	23.5	14.2	15.4	6.4	2.8
Ciprofloxacin	BDL	10	4.3	8.4	35.1	10.6
Erythromycin	BQL	2.6	3.3	20.2	2	1.7
Sulfamethoxazole	1.4	9.3	7.1	12.1	6.7	3.5
β-Blockers						
Atenolol	BDL	2.3	9.6	8.5	BDL	BDL
Propranolol	BDL	BDL	BDL	BDL	BDL	BDL
Hormones						
E1	BDL	BDL	BDL	BDL	BDL	BDL
E2	BDL	1	1.2	BDL	BDL	1
E3	4.8	2.2	3.2	2.4	4.2	3.9
EE2	BDL	BDL	BDL	BDL	BDL	BDL
NSAIDs						
Diclofenac	5.3	5.6	1.4	6.5	7.2	3.7
Ibuprofen	BDL	53.3	147.5	116.6	BDL	BDL
Naproxen	12.1	219	233	473	394	337
Stimulant						
Caffeine	1692	1424	687	354	2035	33.2

All concentrations are in ng/L
BDL: Below Detection Limit
BQL: Below Quantification Limit

NSAIDs and Caffeine had the highest concentrations among other compounds in this sampling term. All pharmaceuticals and hormones except caffeine reached their lowest levels in the lake due to the precipitations. Even though there was vast amount of dilution due to precipitations, caffeine concentrations were still observed at high concentrations except Beylikçayı. These results confirm high caffeine loads flowing into the lake and the rivers.

Measurement results of samples taken in July are provided in Table 4.6.

Table 4.6: Concentrations of pharmaceuticals and hormones in July.

	Lake	Karasu	Tahtakoprü	Hamza	Ahlat	Beylikçayı
Antibiotics						
Amoxicillin	1.82	3.9	1.1	8.4	1654	18.4
Ciprofloxacin	822	322	207	1537	13567	3580
Erythromycin	10.4	16.3	12.9	21.1	131	56.8
Sulfamethoxazole	5.7	38.0	56.4	332	31.4	229
β-Blockers						
Atenolol	BDL	30.1	11.7	BDL	122.3	83.1
Propranolol	129	160	90.4	561	66.5	BDL
Hormones						
E1	5.7	6.0	6.0	BDL	BDL	BDL
E2	10.2	9.9	BDL	9.8	BDL	10.2
E3	BDL	BDL	BDL	BDL	BDL	BDL
EE2	11.7	13.0	BDL	BDL	BDL	14.0
NSAIDs						
Diclofenac	BDL	12.3	BDL	7.0	2.5	30.7
Ibuprofen	BDL	96.1	BDL	BDL	26.7	111
Naproxen	BDL	401	184	203	1298	12300
Stimulant						
Caffeine	1793	256	1446	328	5435	47.8

All concentrations are in ng/L
BDL: Below Detection Limit
BQL: Below Quantification Limit

Highest concentrations of almost all compounds were observed in this sampling term due to dry weather conditions. Ciprofloxacin concentrations in Ahlat and Beylikçayı were close to concentrations measured in hospital effluents (Seifrtova et al., 2008; Verlicchi et al., 2010). Amoxicillin concentration in Ahlat was also unexpectedly high. Higher values, respect to other sampling terms, of erythromycin and sulfamethoxazole were observed in July. E1, E2, and EE2 had 50% detection frequency with higher concentrations respect to other sampling terms. On the other hand, E3 was not detected in July that all of the other sampling terms it was conversely detected in all sampling points. Naproxen concentrations in Ahlat and Beylikçayı are typical wastewater concentrations (Camacho-Munoz et al., 2010; Jelic et al., 2011). It can easily be said that Ahlat and Beylikçayı were the most polluted and wastewater dominated streams with concentrations found typically in wastewaters in this sampling term. High ciprofloxacin and caffeine concentrations in the lake indicate high ciprofloxacin and caffeine load flowing into the lake.

Measurement results of samples taken in October are provided in Table 4.7.

Table 4.7: Concentrations of pharmaceuticals and hormones in October.

	Lake	Karasu	Tahtakoprü	Hamza	Ahlat	Beylikçayı
Antibiotics						
Amoxicillin	BDL	63.9	BDL	57.3	30.3	33.6
Ciprofloxacin	49.3	191	65.3	416	141	110
Erythromycin	1.8	31.4	4.1	6.9	3.7	11.3
Sulfamethoxazole	3.6	85.5	10.9	63.4	10.3	98.9
β-Blockers						
Atenolol	BDL	54.8	9.2	BDL	7.8	13.9
Propranolol	71.6	137	30.5	BDL	BDL	19.3
Hormones						
E1	BDL	BDL	1.92	BDL	BDL	BDL
E2	1.73	BDL	BDL	BDL	BDL	BDL
E3	11.3	9.8	BDL	16	8.8	BDL
EE2	BDL	BDL	BDL	BDL	BDL	BDL
NSAIDs						
Diclofenac	52	45.7	34.7	BDL	BDL	BDL
Ibuprofen	238	209	182	215	263	136
Naproxen	1.4	102	51.1	34.1	28.6	88.3
Stimulant						
Caffeine	442	4160	1257	576	421	4800

All concentrations are in ng/L
BDL: Below Detection Limit
BQL: Below Quantification Limit

Dramatic effects of dry weather conditions were decreased in this term. Still, higher concentrations than winter and spring sampling terms were observed. High ibuprofen concentrations may be due to high usage rates of this pharmaceutical in this time of the year. Hormone levels returned to its condition before summer with low detection rates for E1, E2, and EE2 and high detection rates for E3. Caffeine concentration in the lake was at the lowest state which is sign of decrease in caffeine loads.

Median, maximum concentrations and frequency of quantifications in the lake and its tributaries were provided in Table 4.8.

Table 4.8: Median, maximum concentrations and frequency of quantification of the compounds.

	Lake			Karasu			Tahtakoprü			Hamza			Ahlat			Beylikçayı		
	#[1]	max	median[2]	#[1]	max	median[2]	#[1]	max	median[2]	#[1]	max	median[2]	#[1]	max	median[2]	#[1]	max	median[2]
Antibiotics																		
Amoxicillin	40	4.00	2.91	100	63.9	21.4	80	14.2	6.35	80	57.3	12.3	100	1654	40.6	100	33.6	13.8
Ciprofloxacin	80	822	30.4	80	322	102.9	100	207	7.1	100	1537	8.40	100	13567	44.5	75	3580	110
Erythromycin	60	10.4	1.80	100	31.4	7.1	100	12.9	4.1	100	21.1	6.90	100	131	4.20	75	56.8	11.3
Sulfamethoxazole	80	5.73	2.55	100	85.5	37.98	100	56.4	10.9	100	332	23	100	31.4	10.3	100	229	51.6
β-Blockers																		
Atenolol	40	4.70	2.70	100	54.8	17.1	100	11.7	9.20	60	8.50	3.70	80	122	26.8	50	83.1	48.5
Propranolol	40	129	100	40	160	148	40	90.4	60.4	20	561	561	20	66.5	66.5	25	19.3	19.3
Hormones																		
E1	20	5.74	5.74	40	6.04	3.57	40	6.01	3.97	0	0	0	20	1.40	1.40	0	0	0
E2	80	10.2	1.42	60	9.9	1.30	20	1.20	1.20	40	9.78	5.39	20	2.00	2.00	75	10.2	1.10
E3	80	11.3	4.70	80	9.85	3.15	60	3.70	3.20	80	16.0	2.15	80	8.84	5.20	50	3.90	3.65
EE2	40	11.7	11.65	20	13.1	13.1	0	0	0	0	0	0	0	0	0	25	14.0	14.0
NSAIDs																		
Diclofenac	80	52.0	5.40	100	45.7	7.80	80	34.7	4.10	80	8.30	6.74	60	8.10	7.20	75	30.7	3.70
Ibuprofen	40	238	134	80	209	74.7	60	182	148	80	215	65.4	60	263	113	75	136	111
Naproxen	80	26.1	10.2	100	502	219	100	233	135	100	473	129	100	1298	394	100	12300	374
Stimulant																		
Caffeine	100	1793	1228	100	4160	1290	100	1446	954	100	1107	354	100	20427	2035	100	5525	2424

All concentrations are in ng/L.
[1]Quantification frequency (%)
[2]Median concentration of positive results

Caffeine and sulfamethoxazole were detected in all of the samples. Amoxicillin, ciprofloxacin, erythromycin, atenolol, E3, diclofenac, ibuprofen, and naproxen were detected in most of the samples. EE2 was the least detected compound. Caffeine had the highest median and maximum concentrations. Since caffeine in environmental samples is an indicator for wastewater pollution (Guo and Krasner, 2009), all rivers and lake are thought to have been polluted by wastewater. Highest concentrations of caffeine, ciprofloxacin, and naproxen were observed at µg/L levels. The concentrations of all hormones exceeded the endocrine disrupting level of 1 ng/L (Routledge and Sumpter, 1996) at least once and mostly more than once.

All antibiotics were detected in most of the samples. In most cases, amoxicillin concentrations were higher than other antibiotics which is expected since excretion rate of amoxicillin is between 80-90% (Jjemba, 2006). However, this is not valid for samples taken in July. Ciprofloxacin concentrations were higher than amoxicillin in all sampling points and sulfamethoxazole and erythromycin concentrations were higher than amoxicillin in the lake, Karasu, Tahtakoprü and Hamza Rivers in July. Amoxicillin's photodegradability (Mavronikola et al., 2009) and other antibiotics' persistency may have caused this result during dry weather conditions. In spite of photodegradability of amoxicillin, high detection indicates high discharge of it. Ciprofloxacin concentrations were unexpectedly high (few µg/L) and close to levels observed in hospital effluents (Seifrtova et al., 2008; Verlicchi et al., 2010), in Ahlat, Beylikçayı Creeks, and Hamza River in June. Amoxicillin, erythromycin, and sulfamethoxazole concentrations were in range similar to previous studies.

Detection frequency of propranolol was very low since excretion rate of propranolol as an unchanged product is below 1%. Nevertheless, propranolol was measured as high as 561 ng/L in dry weather conditions. Atenolol was determined in every sampling period as expected from its high usage and about 90% excretion rate as an unchanged compound (Zuccato et al., 2005). Atenolol and propranolol concentrations were similar to previous studies (Bendz et al., 2005; Vieno et al., 2006; Zuccato et al., 2005).

There are conversion mechanisms among E1, E2, and E3. E1 is favored in these mechanisms. However, adsorption rate of E1 to sediments is higher than E2. It is theorized that E2 was converted to E1 meanwhile some of E1 was adsorbed to sediment, some of it converted E3. Therefore, E2 and E3 were the highest detected

compounds among hormones (60% in average) and EE2 and E1 were detected only four and six times in 29 samples, respectively. These results confirm lab scale studies found that E1 and EE2 are more easily removed from aqueous phase than E2 and E3 in field scale. However, it is not valid for sample taken in June with detection of E3 neither of the sampling points. This may be caused by exposure of UV susceptible hormones to higher UV radiation and going under high rate of degradation. Although EE2 is more stable and persistent than natural hormones, low detection frequency reflects low usage rate of this compound. Even though all hormone concentrations were very close to the quantification limit, these concentrations are high enough to induce endocrine disruption in aquatic species in the watershed. Moreover, since concentrations of some hormones in Büyükçekmece Lake, which is an important drinking water source for Istanbul, have reached levels as high as 11.7 ng/L, hormones may pose a threat to human health.

Diclofenac concentrations were in the similar range with previous occurrence studies in surface waters. Ibuprofen concentrations were similar to studies conducted in Luxemburg and South Korea, but higher than in UK, Italy and USA. Naproxen generally had highest concentrations among NSAIDs as generally observed in the literature (Fernandez et al., 2010; Hernando et al., 2006; Hilton and Thomas, 2003; Kasprzyk-Hordern et al., 2008c; Kim et al., 2007a; Pailler et al., 2009; Weigel et al., 2004). On the other hand, in July naproxen concentrations were 1.3 µg/L and 12.3 µg/L in Ahlat and Beylikçayı Creeks, respectively similar to concentrations observed in wastewater (Camacho-Munoz et al., 2010; Jelic et al., 2011).

The maximum concentrations were observed in July and October. The difference between wet weather conditions (winter/spring) and dry weather conditions (summer/fall) was one or two orders of magnitude. Since there were not enough positive results for hormones in order to explain seasonal variations, seasonal changes in median concentrations of only pharmaceuticals were provided in

Figure 4.3.

Highest median concentrations for all pharmaceuticals were measured either in July or October except for caffeine. Most dramatic increases in concentrations during dry weather conditions were observed for ciprofloxacin, propranolol, atenolol, and sulfamethoxazole. The increase in these concentrations in summer sampling period

indicates that wastewater discharges dominate streams during this period. In particular, small streams like Ahlat and Beylikçayı Creeks were affected more from wastewater domination.

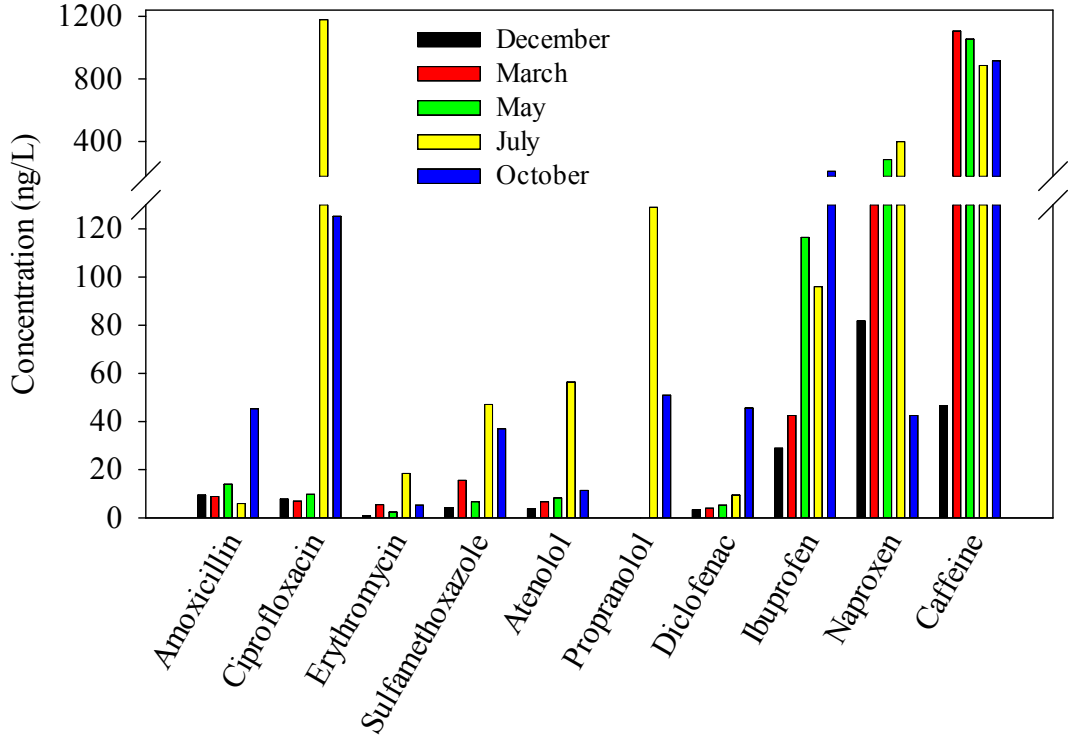


Figure 4.3: Seasonal variations in median concentrations.

Pharmaceutical and water usage rates differ from country to country and even among different communities in a country. However, it is not possible to predict environmental concentrations of PPCPs from usage rates due to the fact that different environmental conditions in a watershed will affect the fate of these compounds. Therefore, the concentrations of pharmaceuticals and hormones must be monitored on a watershed basis.

4.3 Ecotoxicological Test Results

The two sets of data are collected based on ecotoxicological tests on single compounds and ecotoxicological tests on mixtures of compounds within the therapeutic groups. Mixtures of the compounds according to the therapeutic groups were prepared to determine their effect when they are in mixture. Concentrations of compounds used in the mixture were selected based on their single toxicity results and serial dilutions were prepared. Some pharmaceuticals and hormones did not exhibit any effect at water soluble (bioavailable) concentrations in particular in *D.*

magna immobilization test. These compounds were not included in the mixtures to be able to correctly predict the possible additive/synergistic effect.

To figure out the interactions of the compounds, the measured effects of these concentrations in the mixtures were compared with the effect of the same concentrations of each compound when they are single in a solution. The sum of singular effects of each compound in the mixture was predicted with a model. According to the model, to calculate the sum of singular effects in the mixture the equation below was used.

$$\sum_{i=1}^{n} EC\ c_i \qquad \textbf{(4.1)}$$

where "c_i" represents the individual concentrations of the single substances present in a mixture, and "EC c_i" are the effects of single substances that would alone cause at the concentration "c_i". According to this equation, result should be equal to the measured effect assuming additive response. Consequently, two data sets consisting of the measured and the predicted effects were compared. While results smaller than the measured effect indicate synergistic interaction of the compounds, antagonistic interaction causes a result higher than the measured effect.

4.3.1 *D. magna* acute immobilization test results

Before single compound tests, the range of the working solution concentrations was determined in the light of literature values. The ranges were selected narrow enough to establish a reliable non-linear regression and wide enough to cover certain endpoints such as EC50.

Acute immobilization test results were main data used to select chronic test concentration ranges.

Among antibiotics, ciprofloxacin triggered no acute effect on *D. magna* in the range of bioavailable concentrations (<10 mg/L). Other studied antibiotics are more soluble in water. Therefore, their acute immobilization tests were conducted (Figure 4.4).

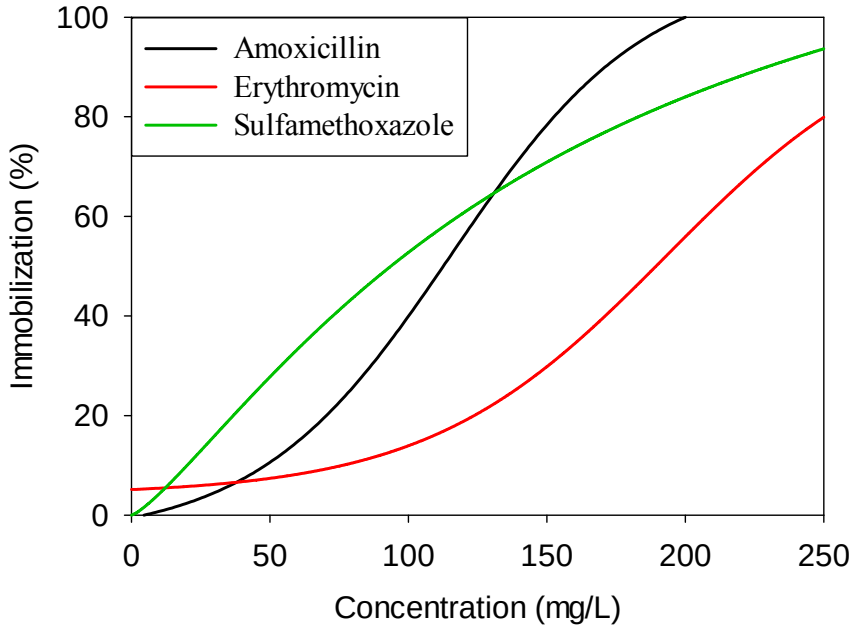


Figure 4.4: Regression curves of antibiotics for immobilization of *D. magna* in 48h.

Although all concentrations studied for acute effects of antibiotics to *D. magna* were much higher than environmental concentrations, this test shows trends of the effects of antibiotics. EC50 values were 113 mg/L, 189 mg/L, and 95 mg/L for amoxicillin, erythromycin and sulfamethoxazole. Although gap between curves of amoxicillin and erythromycin increases with increasing concentrations, they have similar effects at concentrations lower than 50 mg/L. Shape of the curve of sulfamethoxazole and consequently effect trend is different from of which erythromycin and sulfamethoxazole since while 3 parameter logistic non-linear regression was used for sulfamethoxazole 4 parameter sigmoid non-linear regression was used for others. Sulfamethoxazole had the highest effects at concentrations below 125 mg/L which is unlikely to find in environmental waters.

A mixture of these three antibiotics was prepared to determine their effect when they are in mixture. Concentrations in mixture were prepared according to acute toxicity results with an assumption that they will have additive interaction when they are in mixture and serial dilutions were prepared (Table 4.9).

Table 4.9: Concentrations of antibiotics in mixture for *D. magna* immobilization test.

Concentration Level	Amoxicillin	Erythromycin	Sulfamethoxazole
1	6	15	9
2	12	31	18
3	25	62	36
4	50	125	73

All concentrations are in mg/L

The 3 parameter sigmoidal estimations of predicted and measured curves were provided in Figure 4.5.

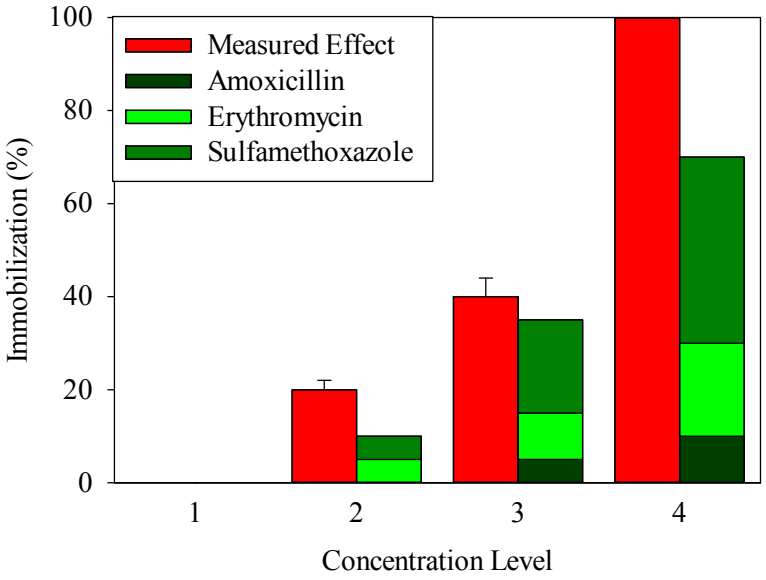


Figure 4.5: Measured and predicted curves for antibiotic mix for *D. magna* immobilization test.

The main finding is antibiotics interact synergistically when they are in mixture. The differences between measured and predicted effects are limited until concentration level 3. After that, the gap between the effects increases indicating synergistic interaction increases.

Atenolol and propranolol are the β-blockers that were tested (Figure 4.6).

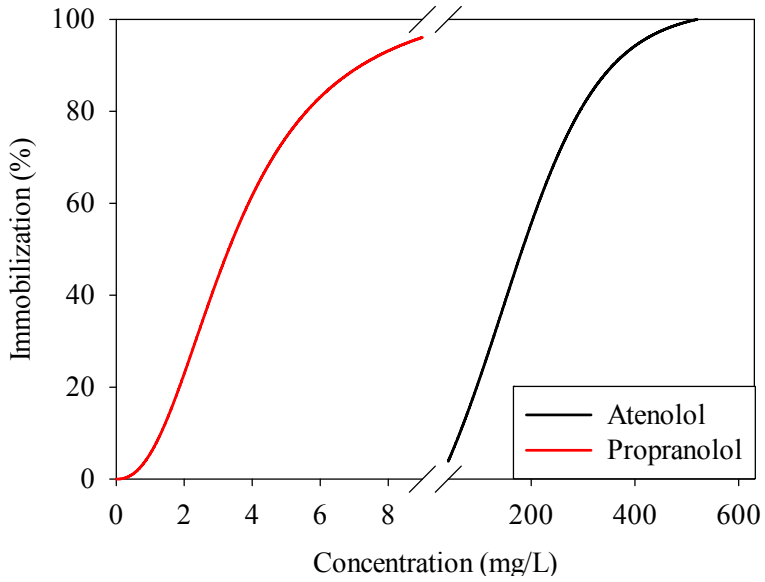


Figure 4.6: Regression curves of β-blockers for immobilization of *D. magna* in 48h.

Even though atenolol and propranolol belong to same therapeutic group, their acute impacts to *D. magna* are very different. While EC50 of atenolol was 185 mg/L, propranolol had 3.3 mg/L. Atenolol and propranolol had 32 mg/L and 1.24 mg/L NOECs, respectively. While atenolol had 295 mg/L EC80 value it was 5.6 mg/L for propranolol. 4 parameter sigmoid regression for atenolol and 3 parameter logistic regression for propranolol were used to estimate concentration response curves. Although concentration ranges are as different as one order of magnitude, concentration response curves of both atenolol and propranolol have similar shapes. This indicates that these two compounds have similar effect trends to acute immobilization of *D. magna*. Similar EC50 values were reported in previous studies for both atenolol and propranolol (Cleuvers, 2003; 2005; Huggett et al., 2002).

A binary mixture of two β-blockers was prepared to determine their effect when they are in mixture. Concentrations in mixture were prepared according to acute toxicity results with an assumption that they will have additive interaction when they are in mixture and serial dilutions were prepared (Table 4.9).

Table 4.10: Concentrations of β-blockers in mixture for *D. magna* immobilization test.

Concentration Level	Atenolol	Propranolol
1	16	0.62
2	32	1.24
3	65	2.5
4	130	5

All concentrations are in mg/L.

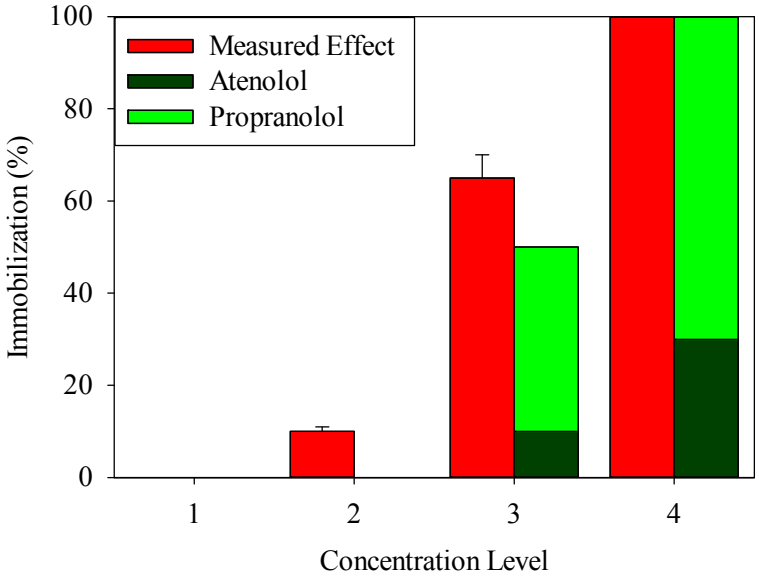


Figure 4.7: Measured and predicted curves for β-blockers mix for *D. magna* immobilization test

The most interesting result for β-blocker mix test was at concentration level 2. At concentration level 1 no effect was observed in mixture as predicted from single effects of the compounds. Even though NOECs of two compounds mixed at concentration level 2, 10% immobilization observed. This situation was just a simple example for presenting how interaction between chemicals may cause drastic and unexpected effects to living organisms. In all points, synergistic interactions were observed.

Naproxen presented no acute effect at bioavailable concentrations to *D. magna* in 48h. Therefore, only for diclofenac and ibuprofen regression curves were estimated as 3 parameter sigmoid and 3 parameter logistic, respectively (Figure 4.8).

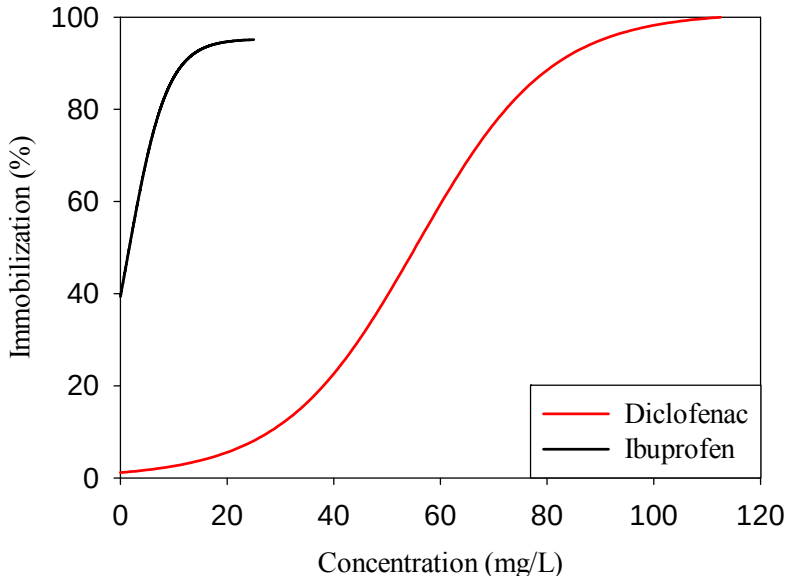


Figure 4.8: Regression curves of NSAIDs for immobilization of *D. magna* in 48h.

Ibuprofen had higher acute effects to *D. magna* than Diclofenac. EC 50 values were 55 mg/L and 1.8 mg/L for diclofenac and ibuprofen, respectively. Ibuprofen's accelerated increase results 7.2 mg/L EC80 value. EC80 for diclofenac was 10 times higher (72 mg/L). NOECs were 4.5 mg/L and 0.35 mg/L for diclofenac and ibuprofen, respectively. Accelerated increase of ibuprofen concentration response curve results in narrow range between NOEC and EC80 (7 mg/L). It is contrary for diclofenac with a 68 mg/L range between NOEC and EC80. Different EC50 values (in 22 mg/L – 108 mg/L range) were reported in different studies for diclofenac in the literature (Cleuvers, 2003; Cleuvers, 2004). The EC50 value found in this study is in this range. However higher EC50 values were reported for ibuprofen from 10 to 100 mg/L (Heckmann et al., 2007).

A binary mixture of diclofenac and ibuprofen and series of dilutions of this mixture were prepared according to single toxicity results (Table 4.11).

Table 4.11: Concentrations of NSAIDs in mixture for *D. magna* immobilization test.

Concentration Level	Diclofenac	Ibuprofen
1	4.5	0.35
2	9	0.7
3	18	1.4
4	36	3.3
5	72	6.6

All concentrations are in mg/L.

Comparison between predicted and measured results for mixture toxicity was provided in Figure 4.9.

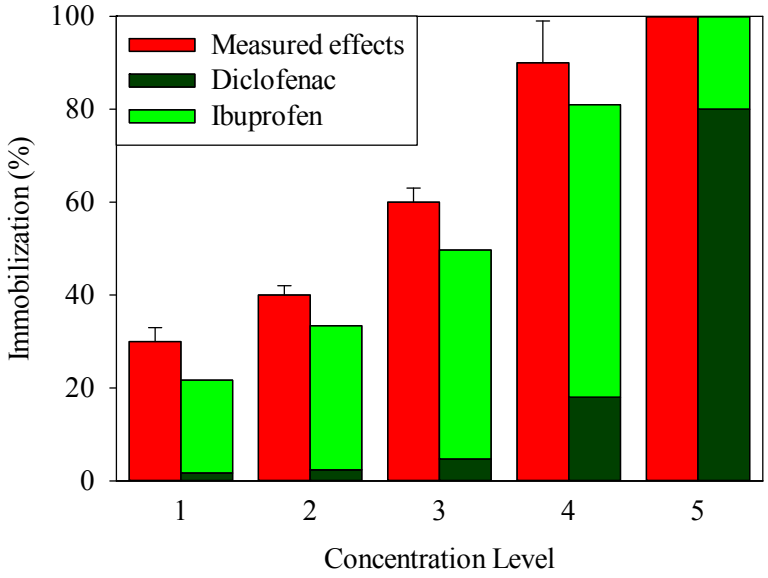


Figure 4.9: Measured and predicted effects of NSAID mixture to immobilization of *D. magna*.

Measured effect bars represent immobilization of *D. magna* at all concentration levels with standard deviation as error bars. Stacked bars represent predicted effects of single compounds in the mixture. At concentration levels 1-3 synergistic interactions were observed. Although, at concentration level 4, it seems that there is a synergistic interaction, residual is close to zero. The gap between measured and predicted effects remains nearly the same at concentration level 1 to 4. At concentration level 5, predicted immobilization was higher than 100% which yielded 100% immobilization in the real case. Diclofenac's contribution to predicted effect remained at low levels for the first three concentration levels since diclofenac's single toxicity regression curve has exponential increase shape until 40 mg/L.

EC50 of caffeine for this test was 206 mg/L (Figure 4.10). NOEC of caffeine was 50 mg/L which is unlikely to be found in environmental waters.

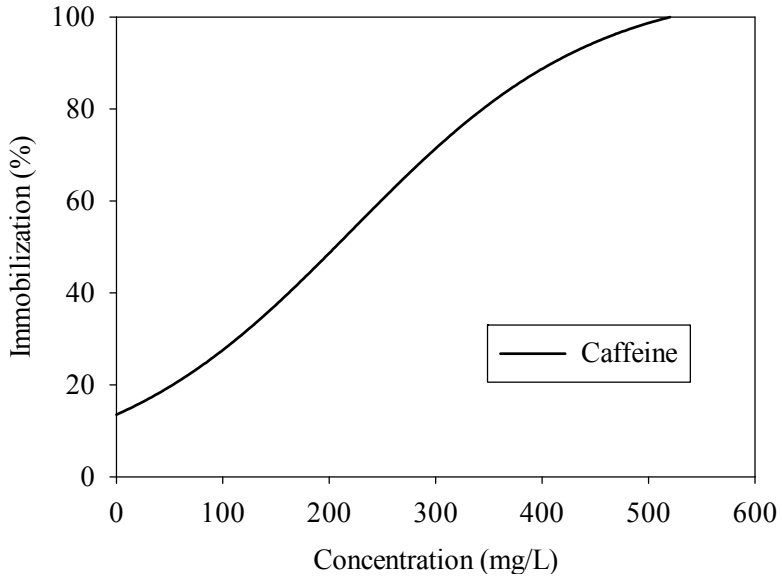


Figure 4.10: Regression curve of caffeine for immobilization of *D. magna* in 48h.

None of the hormones caused immobilization enough to calculate EC endpoints and estimate concentration response curves to *D. magna* at bioavailable concentrations. However, low level immobilizations (<15%) were observed. Therefore, NOECs were calculated as 0.5 mg/L, 0.25 mg/L, 0.16 mg/L and 1 mg/L for E1, E2, E3, and EE2, respectively.

4.3.2 Freshwater algae growth inhibition test results

P. subcapitata are widely used test organisms to determine ecotoxicological effects of chemicals. They are also widely found in freshwater all over the world. Therefore, ecotoxicological data obtained from this test applicable to most of the areas. Since *P. subcapitata* are primary producers, any effect to them would directly affect whole food web. Moreover, their sensitive nature makes them great test species for ecotoxicological bioassays.

All antibiotics were tested in concentration ranges wide enough to cover endpoints and narrow enough to achieve robust non-linear regression estimations (Figure 4.11).

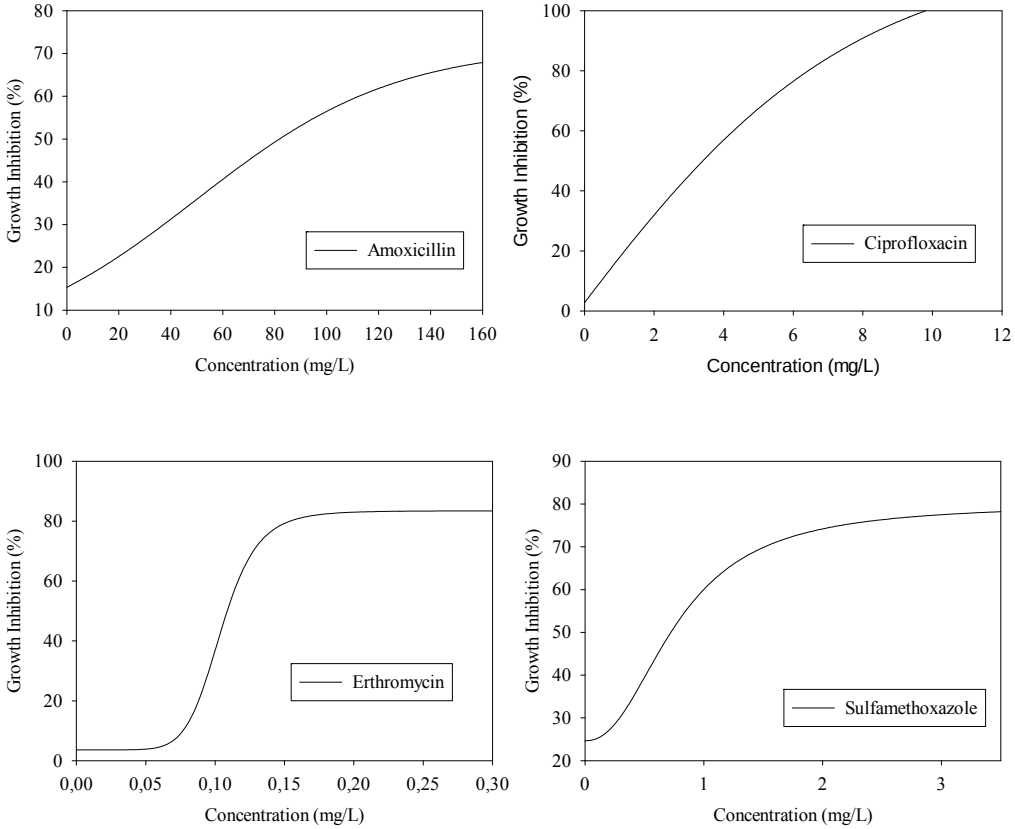


Figure 4.11: Regression curves of antibiotics for freshwater algae test.

Erythromycin had highest impact with 0.11 mg/L, 0.15 mg/L and 0.014 mg/L EC50, EC80, and NOEC values. After erythromycin, sulfamethoxazole comes with 0.72 mg/L EC50 and 0.05 mg/L NOEC. Unlike *D. magna* immobilization test, there was positive result for ciprofloxacin with 3.4 mg/L and 0.1 mg/L EC50 and NOEC, respectively. Amoxicillin triggered lowest impact to *P. subcapitata* with 82 mg/L EC50 value. 3 parameter sigmoid, 4 parameter sigmoid, 4 parameter Hill, 4 parameter logistic non-linear regressions were used to estimate concentration-response curves of amoxicillin, ciprofloxacin, erythromycin, and sulfamethoxazole, respectively. Since all of the concentration-response curves were fit to different non-linear regression method, trends were different for all of the compounds. Since erythromycin causes growth inhibition at low concentrations, its rapidly increasing curve causes only 0.14 mg/L difference between NOEC and EC80. For amoxicillin higher NOEC (250 mg/L) was reported in the literature (Lutzhoft et al., 1999). This may be caused by the photodegradability of amoxicillin. On the other hand, similar EC50 and NOECs were reported for both erythromycin and sulfamethoxazole (Eguchi et al., 2004; Isidori et al., 2005b).

A mixture of amoxicillin, ciprofloxacin, erythromycin, and sulfamethoxazole and series of dilutions of this mixture were prepared according to single toxicity results (Table 4.12).

Table 4.12: Concentrations of antibiotics in mixture for *P. subcapitata* growth inhibition tests.

Concentration Level	Amoxicillin	Ciprofloxacin	Erythromycin	Sulfamethoxazole
1	5	0.1	0.014	0.05
2	10	1	0.028	0.1
3	20	2	0.056	0.2
4	40	4	0.112	0.5

All concentrations are in mg/L.

Comparison between predicted and measured results for mixture toxicity was provided in Figure 4.12.

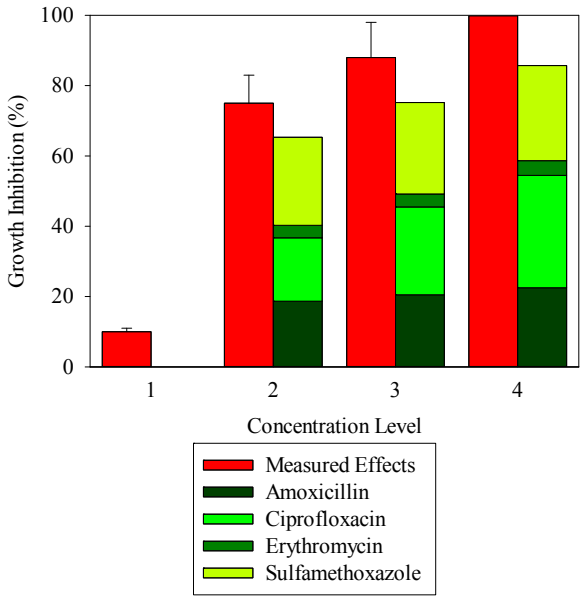


Figure 4.12: Measured and predicted effects of antibiotic mixture to *P. subcapitata*.

At concentration level 1, all antibiotics were mixed at concentrations triggering no effect when they are in mixture. However, 10% growth inhibition was observed at this concentration level. At all concentration levels, measured effects seem higher than predicted effects indicating studied antibiotics interact synergistically. However, at concentration levels 2 and 3 the measured effects and the predicted effects are not statistically different.

β-blockers, atenolol and propranolol, were tested to find growth inhibition to *P. subcapitata* (Figure 4.13).

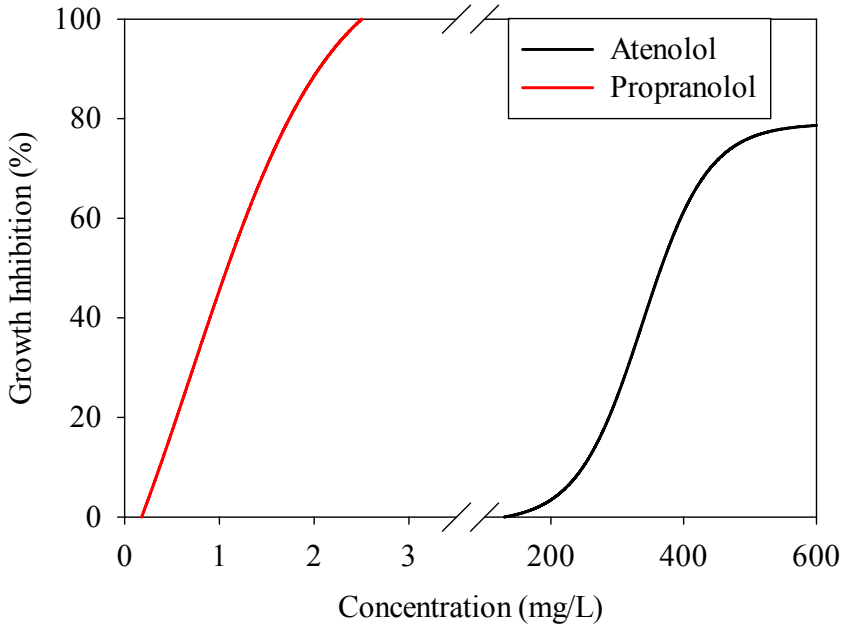


Figure 4.13: Freshwater algae test results for β-blockers.

Like *D. magna* immobilization test atenolol and propranolol had very different effects to growth inhibition of *P. subcapitata* with 367 mg/L and 1 mg/L EC50 values, respectively. While NOECs were 130 mg/L and 0.16 mg/L, EC80s were 600 mg/L and 1.74 mg/L for atenolol and propranolol, respectively. Both of the atenolol and propranolol concentration-response curves were estimated with 4 parameter sigmoid non-linear regression.

A binary mixture of atenolol and propranolol and series of dilutions of this mixture were prepared according to single toxicity results (**Table 4.13**).

Table 4.13: Concentrations of β-blockers in mixture for *P. subcapitata* growth inhibition tests.

Concentration Level	Atenolol	Propranolol
1	100	0.16
2	200	0.31
3	300	0.62
4	400	1.24

All concentrations are in mg/L.

Comparison between predicted and measured results for mixture toxicity was provided in Figure 4.14.

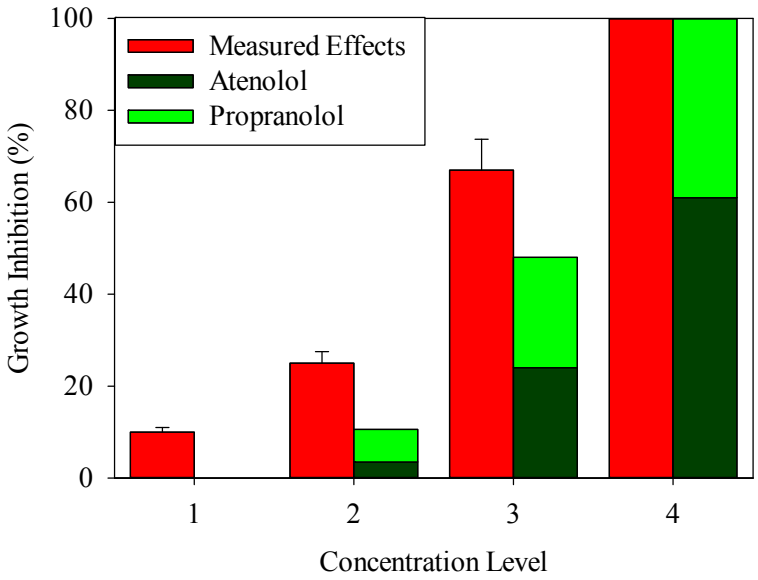


Figure 4.14: Predicted and measured curves for β-blockers.

At concentration level 1, which is prepared with β-blockers at concentrations creating no effects on growth inhibition of *P. subcapitata*, 10% inhibition was observed. At all concentration levels measured effects were higher than predicted effects indicating synergistic interaction between β-blockers.

Unlike *D. magna* immobilization test, hormones triggered adverse effects to growth inhibition of *P. subcapitata* (Figure 4.15).

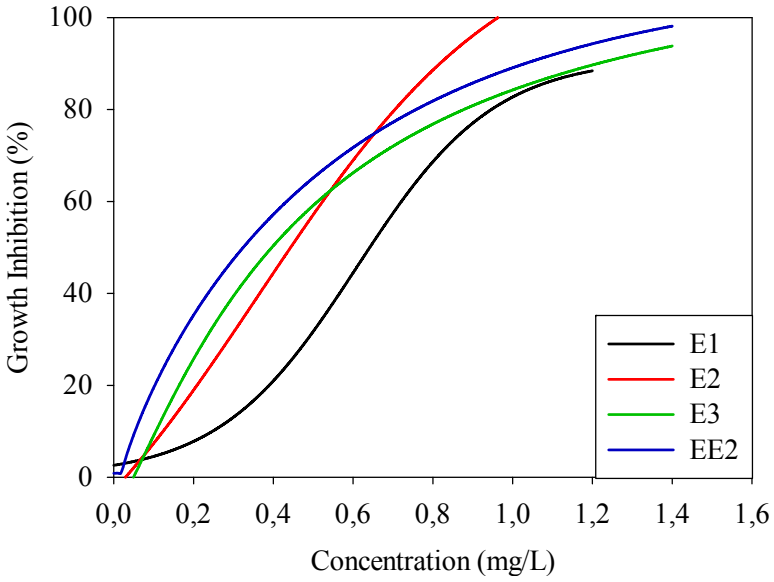


Figure 4.15: Freshwater algae test results for hormones.

All of the hormones had similar effects to *P. subcapitata*. Even though non-linear estimations for all of the compounds are not the same (4 parameter sigmoid for E1 and E2, 3 parameter logistic for E3, and EE2), their trends are similar. EC50s were

0.64 mg/L, 0.45 mg/L, 0.40 mg/L, and 0.32 mg/L for E1, E2, E3, and EE2, respectively. EE2 were the most ecotoxic compound among hormones in concentration range until 0.6 mg/L for freshwater algae growth inhibition test. After 0.6 mg/L, E2 was the most ecotoxic compound. E1 was the least ecotoxic compound among hormones in tested concentration ranges. NOECs were 0.03 mg/L for E1 and E2, 0.04 for E3 and 0.02 for EE2.

A mixture of E1, E2, E3, and EE2 and series of dilutions of this mixture were prepared according to single toxicity results (Table 4.14).

Table 4.14: Concentrations of hormones in mixture for *P. subcapitata* growth inhibition tests.

Concentration Level	E1	E2	E3	EE2
1	30	30	40	20
2	60	60	80	40
3	120	120	160	80
4	250	250	320	160

All concentrations are in µg/L.

Comparison between predicted and measured results for mixture toxicity was provided in Figure 4.16.

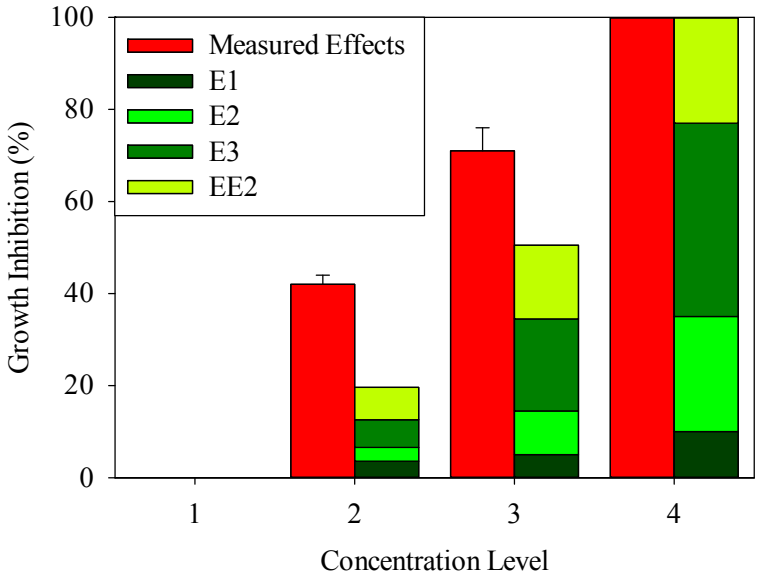


Figure 4.16: Measured and predicted effects of hormone mixture to *P. subcapitata*.

At concentration level 1, all hormones were mixed using NOECs when they are single. No effects were observed at concentration level 1 as predicted. However, at other concentration levels measured effects were more than predicted effects

indicating synergistic interaction among tested hormones at concentrations higher than no effect concentrations.

Concentration-response curves for NSAIDs were estimated using 4 parameter sigmoid non-linear regression for diclofenac and naproxen and 4 parameter weibull non-linear regression for ibuprofen (Figure 4.17).

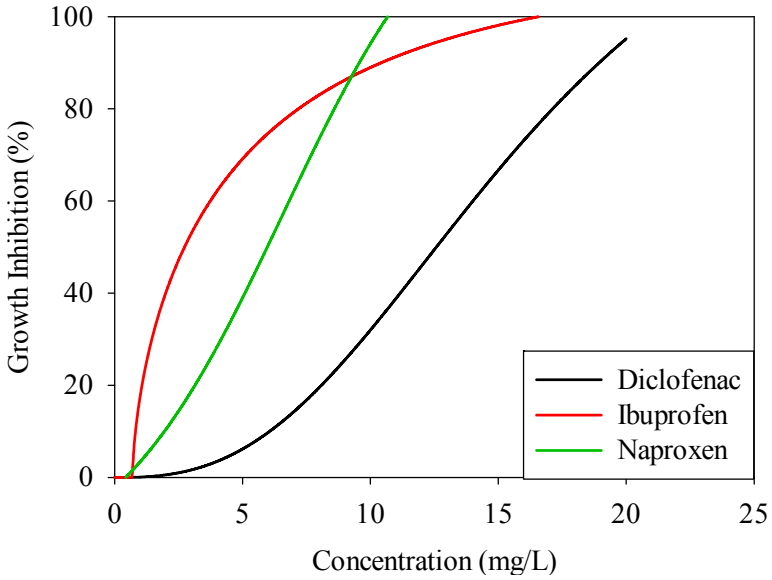


Figure 4.17: Freshwater algae test results for NSAIDs.

Among NSAIDs ibuprofen had highest impact to growth inhibition of *P. subcapitata* at concentrations higher than NOEC (0.7 mg/L) with 2.7 mg/L EC50. Since NOEC of naproxen is 0.35 mg/L, impact of naproxen between 0.35 mg/L and 0.7 mg/L is higher than ibuprofen. EC50 of naproxen was found 5.8 mg/L. Rapidly increasing trend of concentration-response curve of ibuprofen also causes small difference between endpoints as well as higher impact at concentrations higher than NOEC. Diclofenac caused lowest impact to growth inhibition of *P. subcapitata* among NSAIDs with 2.4 mg/L NOEC and 12.5 mg/L EC50. Trend of the concentration-response curve of diclofenac has slower increase respect to ibuprofen and naproxen, yielding wider range between endpoints. EC80s were 17.1 mg/L, 7.2 mg/L, and 8.6 mg/L for diclofenac, ibuprofen, and naproxen, respectively.

A mixture of diclofenac, ibuprofen, and naproxen and series of dilutions of this mixture were prepared according to single toxicity results (Table 4.15).

Table 4.15: Concentrations of NSAIDs in mixture for *P. subcapitata* growth inhibition tests.

Concentration Level	Diclofenac	Ibuprofen	Naproxen
1	0.8	0.7	0.35
2	2	1.9	0.9
3	4	3.8	1.8
4	6	5.5	2.65

All concentrations are in mg/L.

Comparison between predicted and measured results for mixture toxicity was provided in Figure 4.18.

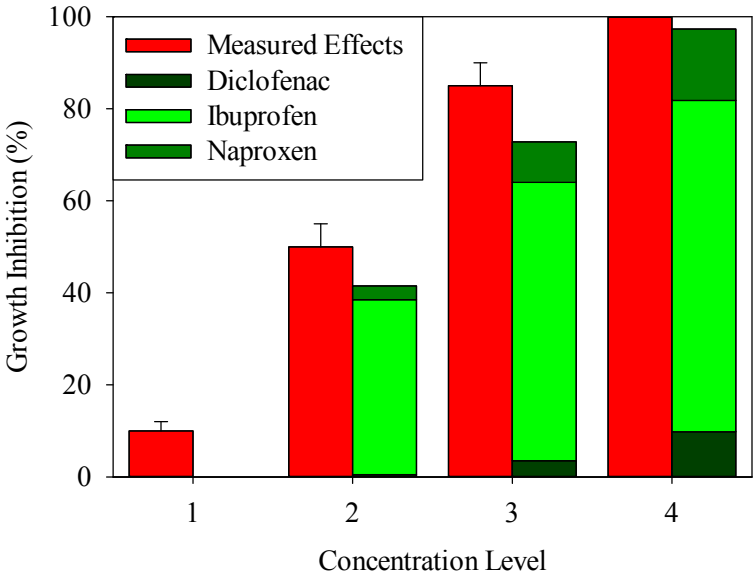


Figure 4.18: Measured and predicted effects of NSAID mixture to *P. subcapitata*.

Concentration level 1 was prepared with NSAIDs at concentrations causing no effect to growth inhibition of *P. subcapitata*. However, 10% growth inhibition was observed. At all concentration levels measured effects were higher than predicted effects indicating synergistic interactions among NSAIDs. Ibuprofen's dominance was predicted in mixtures since it is measured the most ecotoxic compound among NSIADs. Approximately 15% difference (synergy) was observed at concentration levels 3 and 4.

Concentration-response curve for *P. subcapitata* growth inhibition test of caffeine was estimated using 4-parameter sigmoid non-linear regression method (Figure 4.19).

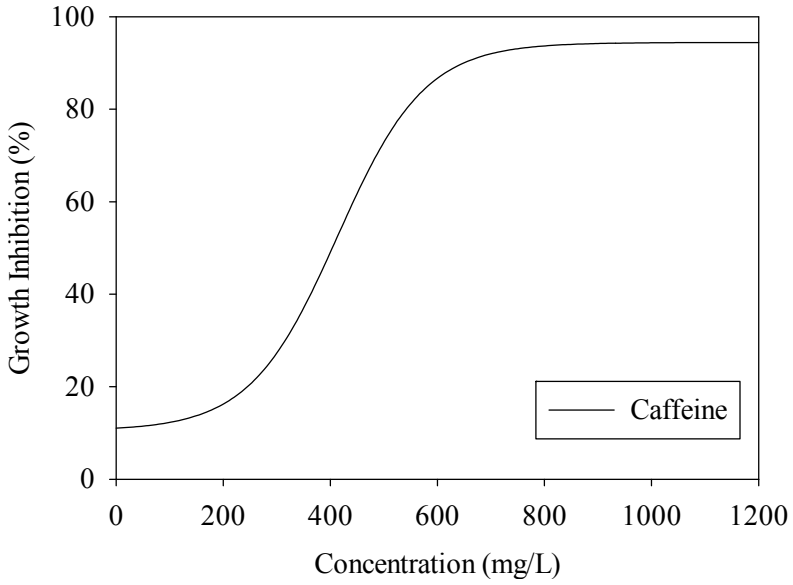


Figure 4.19: Freshwater algae test results for caffeine.

EC10, EC50, and EC80 were 100 mg/L, 405 mg/L and 542 mg/L, respectively. These concentrations were unlikely to be found in environmental waters.

4.3.3 *D. magna* reproduction inhibition test results

D. magna reproduction inhibition test was one of the standard chronic ecotoxicological tests. Although this test was generally not preferred among scientific community due to difficulty to implement 21-day test, it provides important information on chronic effects of compounds to ecosystem.

Since this is a chronic toxicity test, concentrations used in the test are at least one order of magnitude lower than acute toxicity tests.

Concentration-response curves of reproduction inhibition test were estimated using 3-parameter sigmoid non-linear regression for amoxicillin, 4-parameter logistic non-linear regression for ciprofloxacin, and 3-parameter logistic for erythromycin and sulfamethoxazole (Figure 4.20).

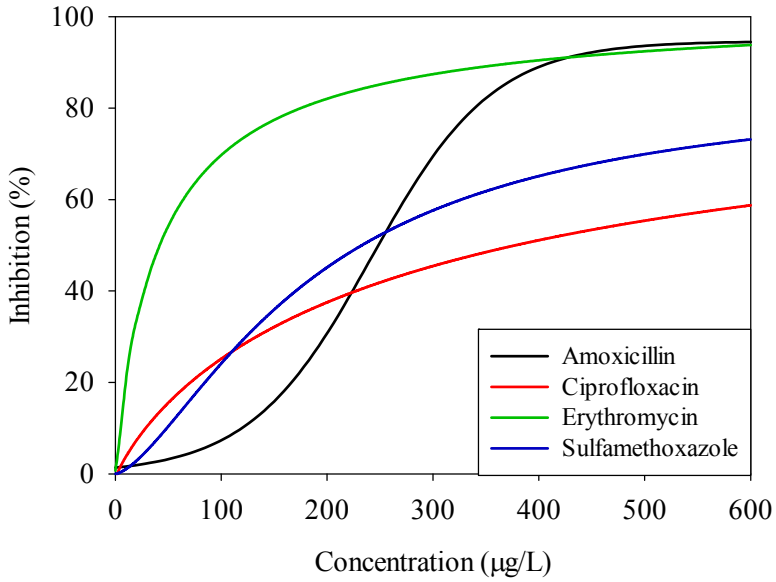


Figure 4.20: *D. magna* reproduction inhibition test results for antibiotics.

Erythromycin caused highest inhibition to reproduction of *D. magna* with 2 µg/L NOEC, 45 µg/L EC50, and 180 µg/L EC80. Moreover, rapidly increasing concentration-response curve of amoxicillin indicate high impacts may be caused at rather lower concentrations. Curves of ciprofloxacin and sulfamethoxazole have an intersection at 100 µg/L. Lower than 100 µg/L ciprofloxacin had higher impact with 2 µg/L NOEC and 30 µg/L EC10 than sulfamethoxazole having 5 µg/L NOEC and 50 µg/L EC10. After 100 µg/L, sulfamethoxazole had higher impact than ciprofloxacin having 376 µg/L EC50. Amoxicillin and sulfamethoxazole curves have an intersection close to EC50 endpoint resulting in similar EC50 values (248 µg/L for amoxicillin and 233 µg/L for sulfamethoxazole). However, different trends of the curves of amoxicillin and sulfamethoxazole indicate lower impacts of amoxicillin at concentrations lower than 250 µg/L, higher impacts at higher concentrations. Amoxicillin had 6 µg/L NOEC and 340 µg/L EC80. Amoxicillin and erythromycin had similar impacts when both of them are more than 350 µg/L.

A mixture of amoxicillin, ciprofloxacin, erythromycin, and sulfamethoxazole and series of dilutions of this mixture were prepared according to single toxicity results (Table 4.16).

Table 4.16: Concentrations of antibiotics in mixture for *D. magna* reproduction inhibition tests.

Concentration Level	Amoxicillin	Ciprofloxacin	Erythromycin	Sulfamethoxazole
1	2	2	2	2
2	10	10	10	10
3	20	20	20	20
4	50	50	50	50

All concentrations are in µg/L.

Comparison between predicted and measured results for mixture effects of antibiotics was provided in Figure 4.21.

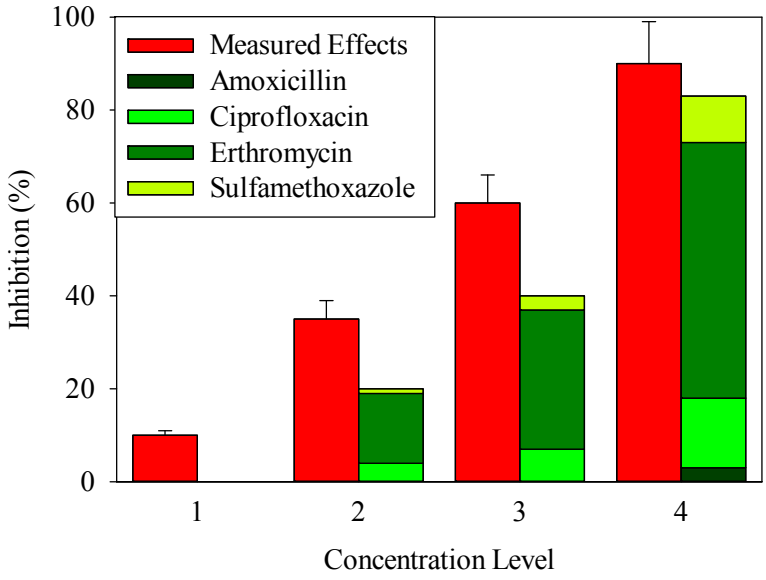


Figure 4.21: *D. magna* reproduction inhibition test results for antibiotic mixtures.

At concentration level 1, all antibiotics were mixed at concentrations having no effect to reproduction of *D. magna*. However, 10% inhibition was observed. Moreover, all antibiotics were 2 µg/L which is quite common in wastewater and even in surface water, at concentration level 1. At concentration levels 2 and 3, measured effects were higher than predicted effects indicating synergistic interaction. At concentration level 4, the measured effect and the predicted effect were not statistically different indicating additive interaction. Synergistic interaction decreases with increasing concentrations from 45% to approximately 0%. These two findings indicate antibiotics may adversely affect ecosystem even when they are low µg/L concentrations.

Concentration-response curves for *D. magna* reproduction inhibition tests were estimated using 3-parameter sigmoid non-linear regression for atenolol and 3-parameter logistic non-linear regression for propranolol (Figure 4.22).

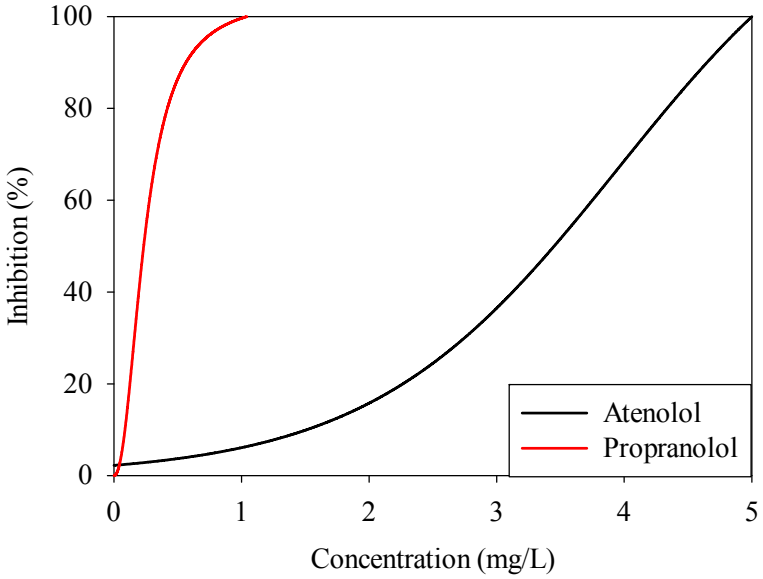


Figure 4.22: *D. magna* reproduction inhibition test results for β-blockers

Impacts of atenolol and propranolol are significantly different for this test as well. Rapidly increasing curve of propranolol represents narrow range of endpoints which are 60 µg/L NOEC, 87 µg/L EC10, 230 µg/L EC50, and 420 µg/L EC80. Since curve of atenolol has exponential type of increase, it covers rather wide concentration range resulting 0.6 mg/L NOEC, 1.51 mg/L EC10, 3.45 mg/L EC50, and 4.33 mg/L EC80.

Atenolol and propranolol concentrations in dilutions prepared for reproduction inhibition test of β-blocker mixture provided in Table 4.17.

Table 4.17: Concentrations of β-blockers in mixture for *D. magna* reproduction inhibition tests.

Concentration Level	Atenolol	Propranolol
1	20	2
2	200	20
3	1000	100
4	2000	200
5	3000	300

All concentrations are in µg/L.

Comparison between predicted and measured results for mixture effects of β-blockers was provided in Figure 4.23.

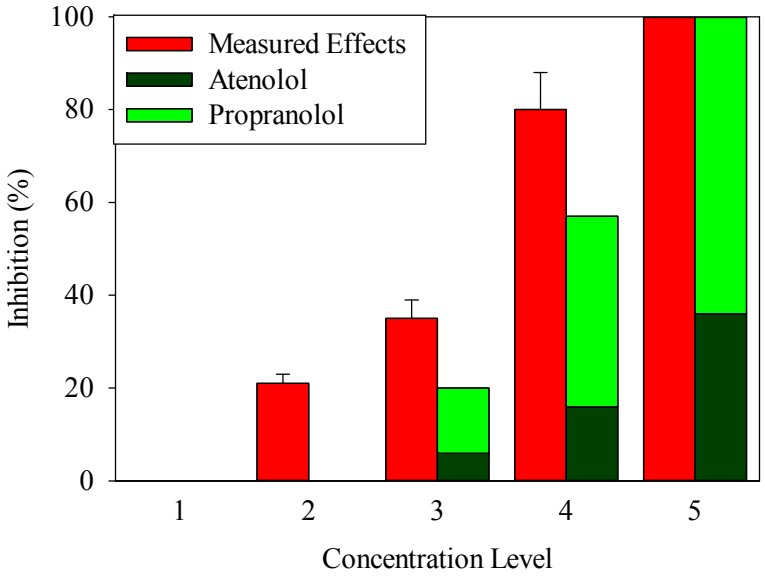


Figure 4.23: *D. magna* reproduction inhibition test results for β-blocker mixtures.

Concentration levels 1 and 2 were prepared with atenolol and propranolol at concentrations having no effect to reproduction of *D. magna* when they are single. At concentration level 1 no effect was detected as predicted. However, at concentration level 2, 20% inhibition was measured. Synergistic interaction decreases along with concentration levels having increasing concentrations as 40% for concentration level 3 and 29% for concentration level 4.

Concentration-response curves were estimated using 3-parameter logistic non-linear regression for E1, E2, and E3 and 3-parameter sigmoid non-linear regression for EE2 (Figure 4.24).

Curve of E2 has the steepest shape causing narrow range between endpoints resulting 0.5 µg/L NOEC, 10 µg/L EC10, 66 µg/L EC50, and 405 µg/L EC80. Curve of E3 has similar shape with curve of E2 but it gets slowly increasing after 100 µg/L. It is found that NOEC is 0.2 µg/L, EC10 is 18 µg/L, EC50 is 283 µg/L for E3. Curve of EE2 has similar shape with curves of E2 and E3. However, rapidly increasing trend starts from relatively higher concentration as well as getting parallel to x axis. NOEC was 0.5 µg/L, EC10 was 17 µg/L, EC50 was 162 µg/L, and EC80 was 269 µg/L for EE2. E1 had lowest impact to reproduction of *D. magna* among hormones with 1 µg/L NOEC, 58 µg/L EC10, 444 µg/L EC50, and 833 µg/L EC80.

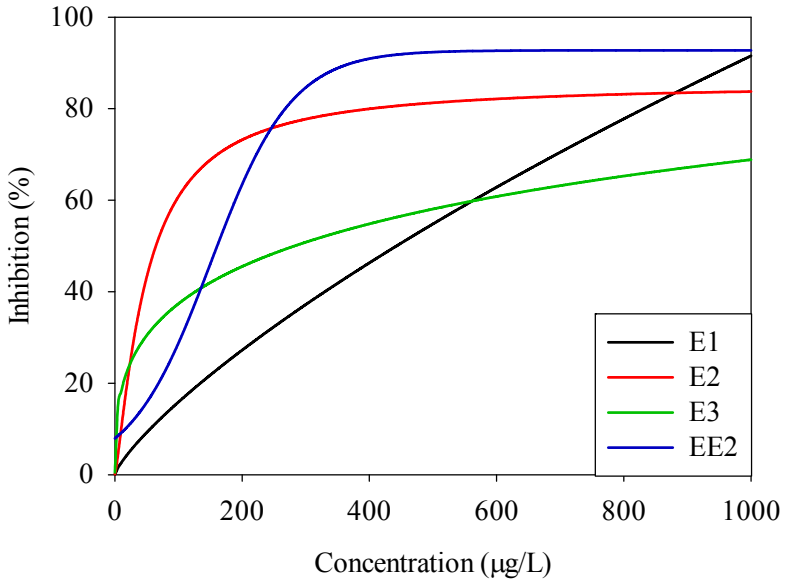


Figure 4.24: *D. magna* reproduction inhibition test results for hormones.

A mixture of hormones and its dilutions were prepared according to single toxicity tests to determine mixture effects of hormones to reproduction of *D. magna* (Table 4.18).

Table 4.18: Concentrations of hormones in mixture for *D. magna* reproduction inhibition tests.

Concentration Level	E1	E2	E3	EE2
1	0.5	0.5	0.2	0.5
2	5	5	5	5
3	20	20	20	20
4	50	50	50	50

All concentrations are in µg/L.

Synergistic interactions were observed for hormone mixtures as well (Figure 4.25).

At concentration level 1, each hormone was mixed using their NOECs. It is predicted that no effect shod have been measured. However, 13% inhibition observed which indicates synergistic interaction occurs among hormones even they would be at NOECs. At concentration levels 2 and 3, 48% and 44% synergistic effects were observed, respectively. At concentration level 2, synergistic effect seems to be 2% due to the measured effect was reached to 100%.

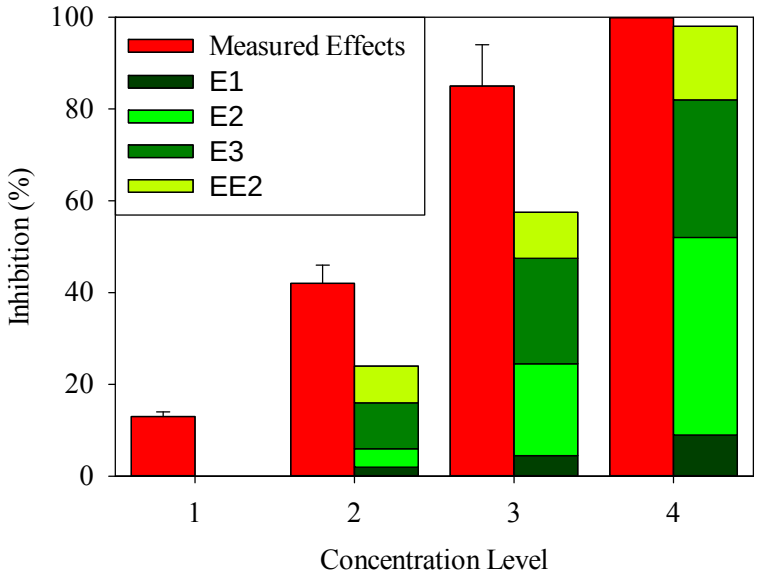


Figure 4.25: *D. magna* reproduction inhibition test results for hormone mixtures.

D. magna reproduction inhibition test curves were estimated using 4-parameter chapman non-linear regression for diclofenac, 3-parameter sigmoid non-linear regression for ibuprofen and naproxen (**Figure 4.26**).

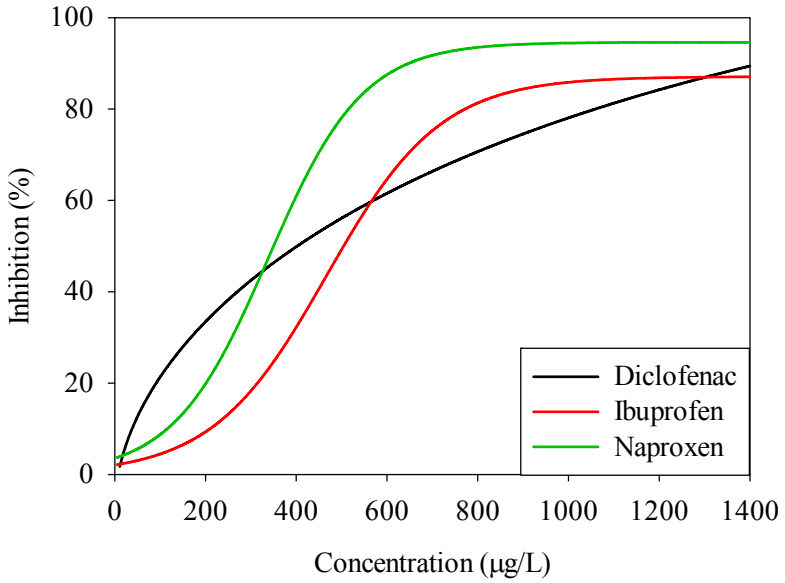


Figure 4.26: *D. magna* reproduction inhibition test results for NSAIDs.

Diclofenac was the most impacted NSAID to reproduction of *D. magna* until 326 µg/L where there is an intersection between curves of diclofenac and naproxen. Therefore, diclofenac had lowest NOEC and EC10 values among NSAIDs as 2.6 µg/L and 40 µg/L, respectively. After that intersection, naproxen is NSAID having highest impact to reproduction of *D. magna* with 350 µg/L EC50 and 515 µg/L EC80. NOEC and EC10 of naproxen were 6 µg/L and 11.5 µg/L, respectively. Ibuprofen

had the lowest impact until 567 µg/L having 8.6 µg/L NOEC, 212 µg/L EC10 and 504 µg/L EC50 which are higher than diclofenac's NOEC (2.6 µg/L), EC10 (40 µg/L), and EC50 (405 µg/L). However, that is inverted for EC80 that are 1058 µg/L and 775 µg/L for diclofenac and ibuprofen, respectively.

A mixture of NSAIDs and serial dilutions for that mixture were prepared to observe mixture effects of NSAIDs to reproduction of *D. magna* (Table 4.19).

Table 4.19: Concentrations of NSAIDs in mixture for *D. magna* reproduction inhibition tests.

Concentration Level	Diclofenac	Ibuprofen	Naproxen
1	2.6	8.6	6
2	26	86	60
3	130	215	150
4	260	430	300

All concentrations are in µg/L.

Synergistic interaction at reproduction inhibition test was observed among NSAIDs (Figure 4.27).

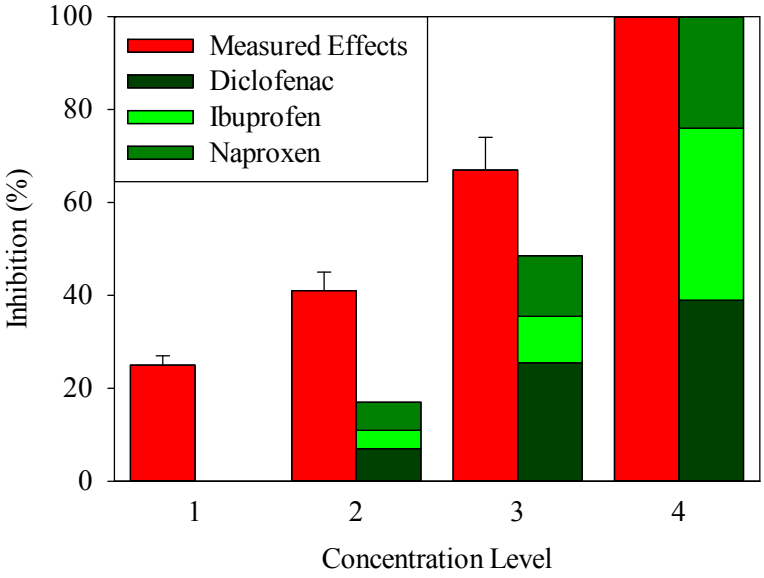


Figure 4.27: *D. magna* reproduction inhibition test results for NSAID mixtures.

At concentration level 1, it was predicted that no effect would have been observed since all NSAID in the mixture were at NOEC. However, 25% inhibition was observed. Synergistic effect decreases from 58% to 28% from concentration level 2 to concentration level 3. More synergistic interaction can be observed in mixture containing lower concentrations of NSAIDs.

Concentration response curve for *D. magna* reproduction inhibition test of caffeine was estimated using 3-parameter logistic non-linear regression method (Figure 4.28).

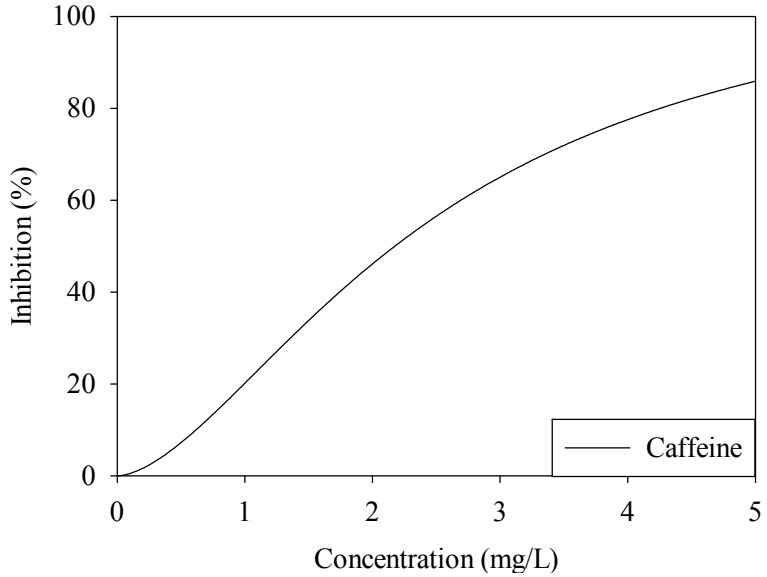


Figure 4.28: *D. magna* reproduction inhibition test results for caffeine.

NOEC, EC10, EC50, and EC80 were 20 µg/L, 610 µg/L, 2.17 mg/L and 4.25 mg/L, respectively.

All compounds were mixed to observe interactive chronic effects when they are in mixture. The concentrations of the compounds in the mixture were selected in the light of the single compound toxicity tests (Table 4.20).

Table 4.20: Concentrations of compounds in total mixture for *D. magna* reproduction test.

Compounds	Concentration Level 1	Concentration Level 2	Concentration Level 3	Concentration Level 4
Amoxicillin	0.02	0.2	1	2
Ciprofloxacin	0.02	0.2	1	2
Erythromycin	0.02	0.2	1	2
Sulfamethoxazole	0.02	0.2	1	2
Atenolol	0.04	0.4	2	4
Propranolol	0.004	0.04	0.2	0.4
E1	0.005	0.05	0.25	0.5
E2	0.005	0.05	0.25	0.5
E3	0.005	0.05	0.25	0.5
EE2	0.005	0.05	0.25	0.5
Diclofenac	0.026	0.26	1.3	2.6
Ibuprofen	0.086	0.86	4.3	8.6
Naproxen	0.06	0.6	3	6
Caffeine	0.2	2	10	20

All concentrations are in µg/L.

Concentration level 4 which contains highest concentrations was prepared with NOECs for each compound. If there were no interaction among the compounds, no effect would have been observed at all of the concentration levels. However, no effect was observed only at concentration level 1. 15%, 19%, and 23% inhibition in reproduction of *D. magna* was observed at concentration level 2, concentration level 3, and concentration level 4, respectively. The concentrations of the compounds in first two concentration levels are very common for environmental waters and wastewaters. Even though the concentrations in the mixtures are below NOECs of the compounds, still they had impact to living organisms and hence ecosystem.

4.3.4 AMES test results

Concentrations of the compounds used for AMES test were selected high enough to see possible mutagenic effects and low enough to prevent inhibition of bacteria used in the test (Table 4.21).

Table 4.21: Concentrations of the compounds used in AMES test.

Compound	Concentration (µg/L)
Antibiotics	
Amoxicillin	1000
Ciprofloxacin	1000
Erythromycin	8
Sulfamethoxazole	1000
B-blockers	
Atenolol	650
Propranolol	60
Hormones	
E1	1000
E2	1000
E3	1000
EE2	600
NSAID	
Diclofenac	1000
Ibuprofen	220
Naproxen	106
Stimulant	
Caffeine	1000

The selected concentrations were higher than possible environmental concentrations to stay at the safe side. Mixtures for each therapeutic group were tested as well. In antibiotic mixture the concentrations of amoxicillin, ciprofloxacin, and sulfamethoxazole were 250 µg/L and 2 µg/L for erythromycin. In β-blocker mixture,

the concentrations of atenolol and propranolol were 325 µg/L and 30 µg/L, respectively. E1, E2, E3, and EE2 concentrations were 250 µg/L, 300 µg/L, 300 µg/L, and 150 µg/L in the hormone mixture, respectively. Diclofenac, ibuprofen, and naproxen concentrations were 300 µg/L, 70 µg/L, and 35 µg/L in the NSAID mixture, respectively.

All wells of all compounds were purple at the beginning of the test as expected (Figure 4.29).

Figure 4.29: Well-plates at the beginning of the AMES test.

General view of the all samples after 5 day incubation period was provided in Figure 42.

After 5 days, some yellow wells indicating mutagenicity were observed (Figure 4.30). However, a statistical analysis, fluctuation test, should be conducted to identify a compound as mutagen.

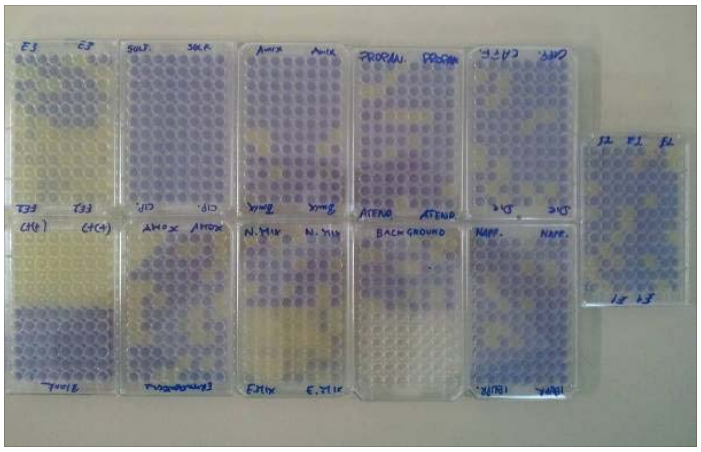


Figure 4.30: Well-plates after 5 days of the AMES test.

While no mutation was observed for blank, all wells of negative control were turned to yellow meaning strong mutagenicity indicating the solutions used in the test contained no mutagen contaminant and the bacteria responding well to a strong mutagen (Figure 4.31).

Figure 4.31: AMES results of blank and positive control.

Some natural (spontaneous) reverse mutations (14 yellow wells) were observed in background test (Figure 4.32).

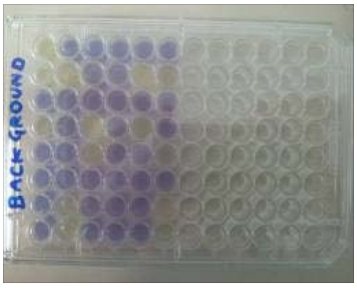


Figure 4.32: AMES results of background.

14 yellow wells in background plate indicate that some spontaneous mutagenicity occurred during the test. This spontaneous mutagenicity was considered as baseline

for tests of compounds and mutagenicity of the compounds were analyzed using fluctuation analysis.

All compounds except ciprofloxacin and sulfamethoxazole had yellow wells in their plates (Figure 4.33).

Yellow wells were counted for each compound (Table 4.22).

Table 4.22: Positive well counts in AMES test.

Compound	Number of Positive Wells
Background	14
Antibiotics	
Amoxicillin	17
Ciprofloxacin	0
Erythromycin	14
Sulfamethoxazole	0
B-blockers	
Atenolol	4
Propranolol	8
Hormones	
E1	10
E2	8
E3	17
EE2	48
NSAID	
Diclofenac	9
Ibuprofen	4
Naproxen	8
Stimulant	
Caffeine	8
Antibiotic mixture	0
B-blocker mixture	9
Hormone mixture	48
NSAID mixture	10

According to fluctuation analysis if background has 14 yellow wells, as it is in this case, there should be at least 24, 28, and 33 yellow wells in sample plates in order to conclude that there is a mutation in 95%, 99% and 99.9% confidence. In this case only EE2 and consecutively hormone mix had mutation effect 99.9% confidence. Other compounds tested and their mixtures do not pose mutagenicity hazard in their environmental concentrations. On the other hand, there were more yellow well in well plates of E3 and Amoxicillin than background. Although they cannot be designated as strong mutagens according to fluctuation test, they may be considered as susceptible compounds.

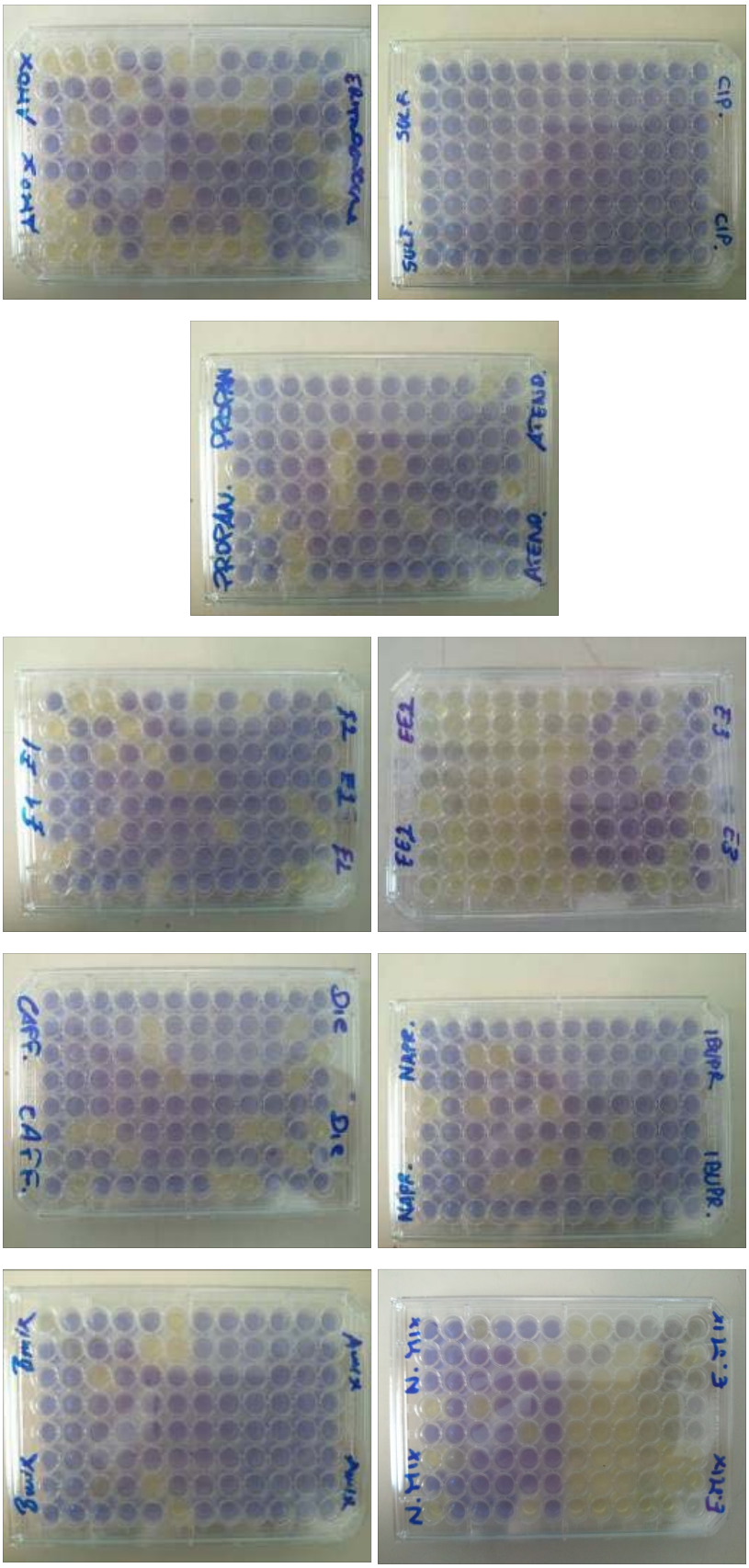


Figure 4.33: AMES test results of the compounds.

4.3.5 YES test R-results

Antibiotics are tested in a concentration range from 50 ng/L to approximately 700 µg/L which can easily be found in environmental waters (Figure 4.34).

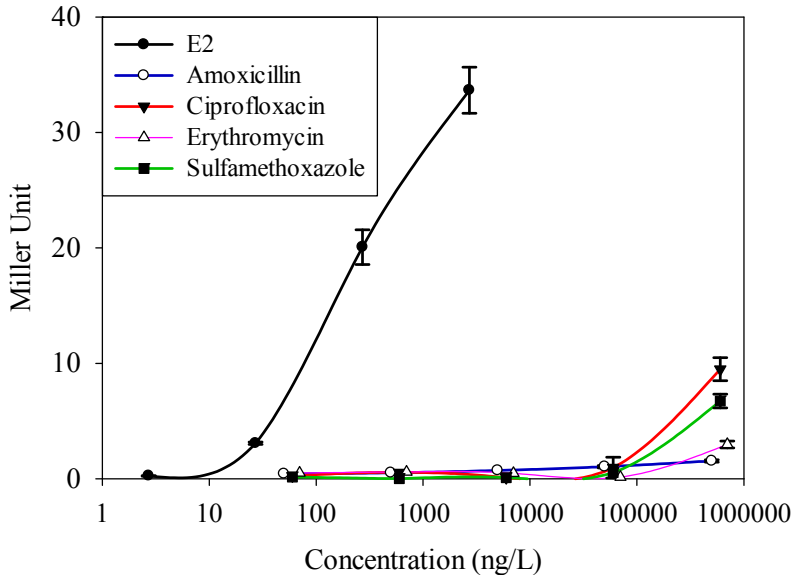


Figure 4.34: YES test results of antibiotics.

Results obtained from the test were compared with the estrogenic effects of E2. No increase in estrogenic effect was observed at low range concentrations for antibiotics. Amoxicillin is the antibiotic which has an estrogenic effect at lowest concentration which is 5 µg/L. However, estrogenicity of amoxicillin does not increase to high levels. Therefore, amoxicillin has low RIE value (5±0.2%). Other three antibiotics' estrogenic effects start at approximately 10 times higher concentration than amoxicillin that 60 µg/L for ciprofloxacin and sulfamethoxazole and 70 µg/L for erythromycin. Ciprofloxacin had the highest RIE value which is 28±3% because of the highest estrogenic effect. Erythromycin and sulfamethoxazole had RIE values as 9±0.1% and 20±1.7%, respectively.

A mixture of antibiotics was prepared to test the estrogenic effects of antibiotics when they are in mixture (Table 4.23).

Table 4.23: Concentrations of the antibiotics in mixture for YES test.

Concentration Level	Amoxicillin	Ciprofloxacin	Erythromycin	Sulfamethoxazole
1	50	60	70	60
2	500	600	700	600
3	5000	6000	7000	6000
4	50000	60000	70000	60000
5	500000	600000	700000	600000

All concentrations are in ng/L.

Concentrations were selected in the light of YES test results of single antibiotics.

The estrogenic effect of antibiotic mixture was very similar to the single estrogenic effects of antibiotics (Figure 4.35).

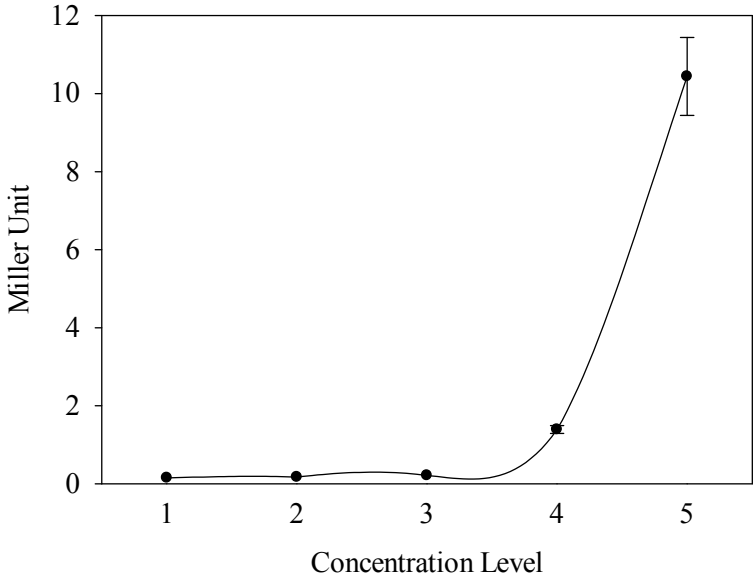


Figure 4.35: YES test results of antibiotic mixture.

Maximum 10.44±1 miller unit estrogenic effect was observed for antibiotic mixture at concentration level 5 that corresponding 31±3% RIE. Although concentration level 5 contains highest concentrations tested of each antibiotic, there is not statistically difference between RIE of antibiotic mixture and RIE of ciprofloxacin. This result indicates no interactive effect occurs among antibiotics for estrogenic effects and estrogenicity is dominated by the antibiotic having highest estrogenic effect.

Both of the β-blockers, atenolol and propranolol, are tested in the concentration range between 50 ng/L and 500 μg/L (Figure 4.36).

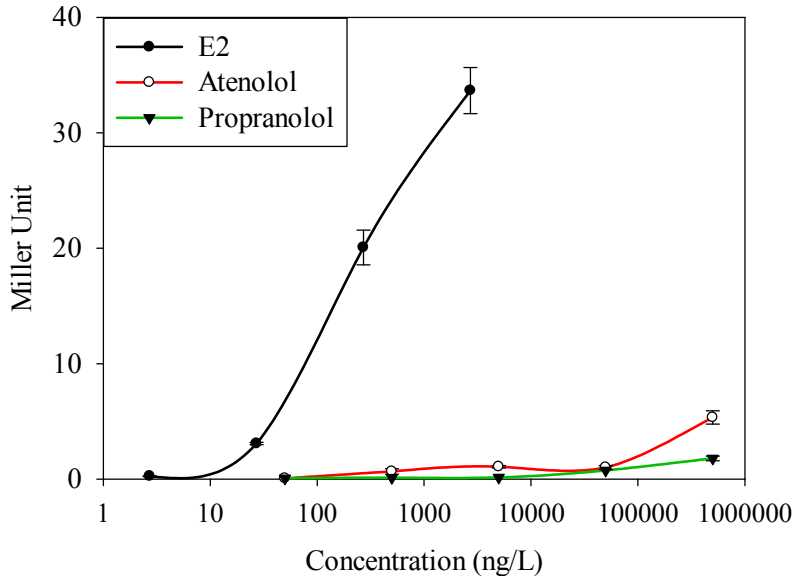


Figure 4.36: YES test results of β-blockers.

Estrogenic effects were observed after 500 ng/L for atenolol and after 5 µg/L for propranolol. Moreover, atenolol had the highest estrogenic effect among β-blockers with 16±2% RIE. Propranolol had 5±0.25 RIE.

In the light of the single estrogenic effect test, a mixture of β-blockers was prepared to measure identify effects (Table 4.24).

Table 4.24: Concentrations of the β-blockers in mixture for YES test.

Concentration Level	Atenolol	Propranolol
1	50	50
2	500	500
3	5000	5000
4	50000	50000
5	500000	500000

All concentrations are in ng/L.

Mixture of β-blockers had estrogenic effect at low level µg/L concentrations (Figure 4.37).

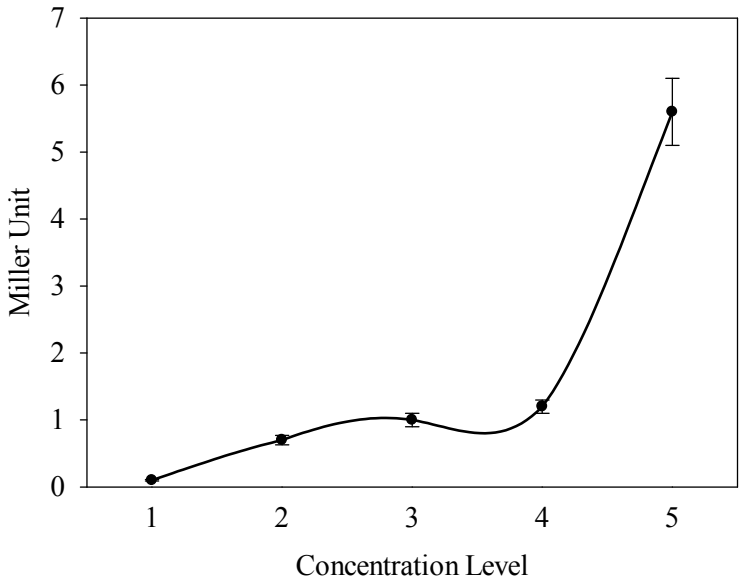


Figure 4.37: YES test results of β-blocker mixture.

Estrogenic effect started to be observed from 2nd concentration level in which there was 500 ng/L atenolol and 500 ng/L propranolol. Actually, shape of the mixture curve was very similar to atenolol curve. Moreover RIE of β-blocker mixture was 17±1 which is not statistically different from RIE of atenolol. This result indicate that highest estrogenic compound dominate estrogenicity of β-blockers when they are in mixture.

NSAIDs were tested to find out their estrogenicity in a concentration range from 55 ng/L to 835 µg/L (Figure 4.38).

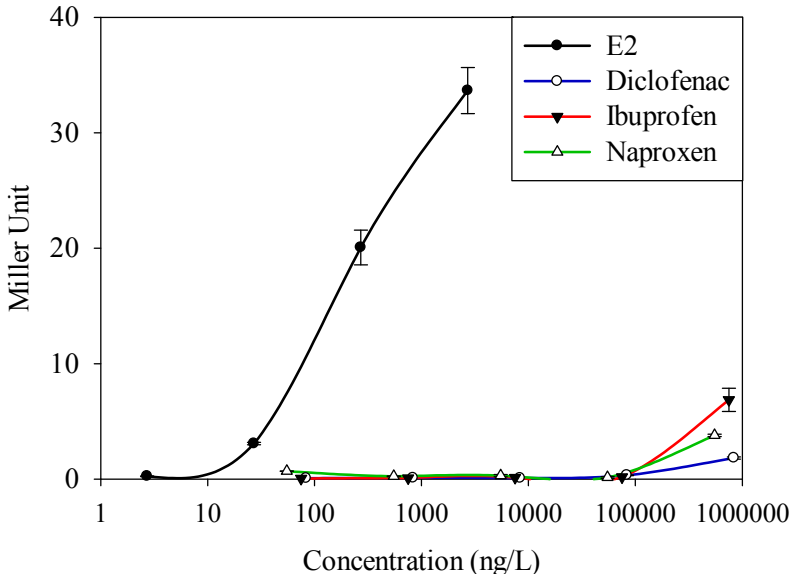


Figure 4.38: YES test results of NSAIDs.

All NSAIDs start to trigger an estrogenic effect after 50 µg/L concentration. Highest impact was observed for ibuprofen with 20±3% RIE. RIE of diclofenac and naproxen are 1.83±0.3 and 3.87±0.3, respectively.

A mixture of NSAIDs and serial dilutions of that mixture were prepared to observe interactive estrogenic effects of NSAIDs (Table 4.25 and Figure 4.39).

Table 4.25: Concentrations of the NSAIDs in mixture for YES test.

Concentration Level	Diclofenac	Ibuprofen	Naproxen
1	83.5	75	55
2	835	750	550
3	8350	7500	5500
4	83500	75000	55000
5	835000	750000	550000

All concentrations are in ng/L.

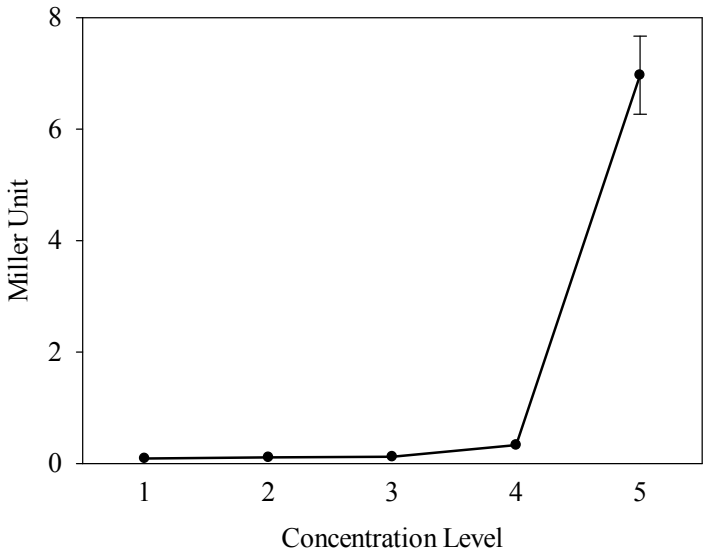


Figure 4.39: YES test results of NSAID mixture.

No estrogenic effect was observed until concentration level 5 for NSAID mixture. At concentration level 5, RIE was calculated as 21±2.2% which is not statistically different from RIE of ibuprofen. The compound triggering highest estrogenic effect for NSIAD mixture (in this case ibuprofen) dominates estrogenic effect of mixture.

Caffeine was tested for its estrogenic effect at concentrations between 125 ng/L and 1.25 mg/L (Figure 4.40).

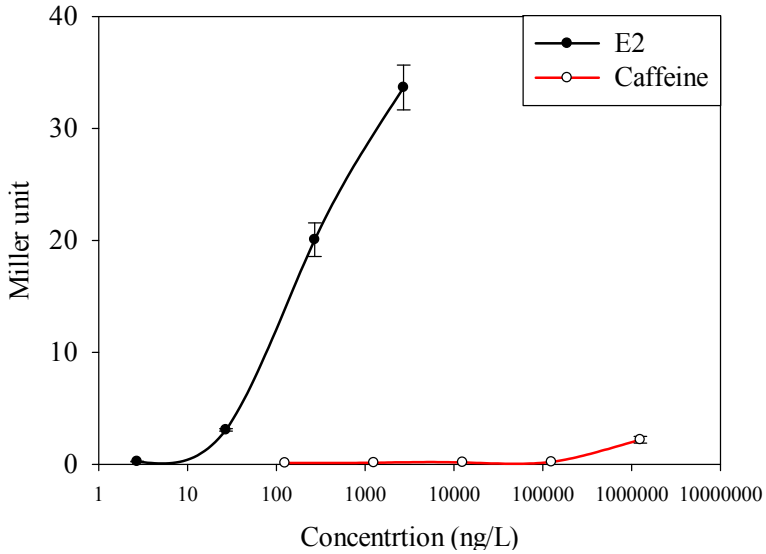


Figure 4.40: YES test results of caffeine.

Caffeine triggers estrogenic effect after 125 µg/L. RIE of caffeine was 7±0.9% at 1.25 mg/L.

All compounds were mixed according to YES test results of single compounds to observe estrogenic effects of the compounds when they are in mixture (Table 4.26).

Table 4.26: Concentrations of the compounds in total mixture for YES test.

Concentration Level	Amoxicillin	Ciprofloxacin	Erythromycin	Sulfamethoxazole	Atenolol	Propranolol	Diclofenac	Ibuprofen	Naproxen	Caffeine
1	50	60	70	60	50	50	83.5	75	55	125
2	500	600	700	600	500	500	835	750	550	1250
3	5000	6000	7000	6000	5000	5000	8350	7500	5500	12500
4	50000	60000	70000	60000	50000	50000	83500	75000	55000	125000
5	500000	600000	700000	600000	500000	500000	835000	750000	550000	1250000

All concentrations are in ng/L.

Estrogenic effect of mixture of all compounds had been observed starting from concentration level 2 (Figure 4.41).

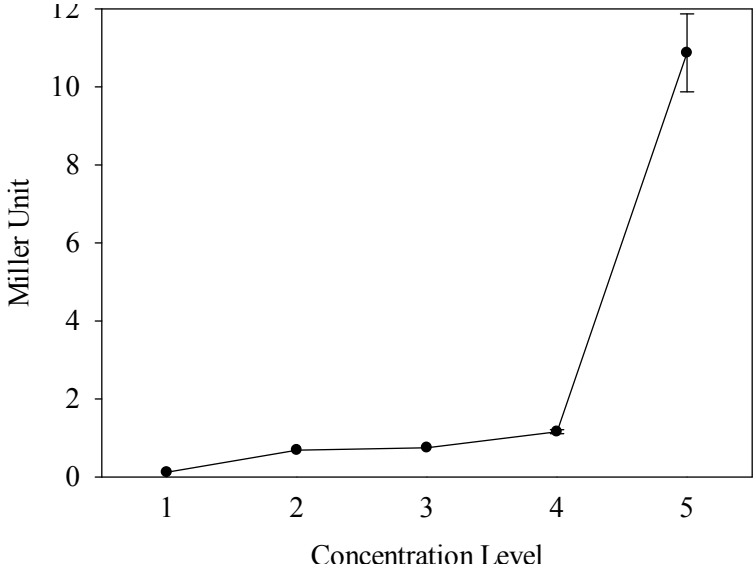


Figure 4.41: YES test results of mixture of all compounds.

Measured effects of mixture of the compounds have some similarities with single estrogenicity tests and estrogenicity tests of therapeutic group mixtures. Estrogenic effect started to be observed at concentration level 2 which was the same as antibiotic mixture ad β-blocker mixture estrogenicity tests. Moreover, at that concentration level atenolol was 500 ng/L of which the estrogenic effect was observed at YES test of atenolol as single compound. Atenolol was the compound showing estrogenic effect at lowest concentration among studied compounds. At concentration level 3, estrogenic effect increases some more where amoxicillin and propranolol were 5 µg/L which is the lowest concentration of them showing estrogenic effect. At concentration level 4, estrogenic effect increases some more and then reaches to its peak at concentration level 5. RIE of mixture of all compounds was found 32.3±3% which is not statistically different from RIE of ciprofloxacin and hence RIE of antibiotics. Domination of the compounds having highest estrogenic effect was also observed for mixture of all compounds at all concentration levels. At concentration level 1, no estrogenic effect was observed. Therefore, concentrations of the compounds at that level can be selected as NOEC of estrogenicity.

5. CONCLUSIONS

Although pharmaceuticals are very important for the protection of human health, they may cause adverse effects in several organisms once they are discharged into the environment. Since pharmaceuticals are designed to exert biological effects, it is expected that they adversely affect ecosystem. Moreover, they may pose threat to human health via food web and/or direct exposure. Therefore, pharmaceuticals in the ecosystem must be monitored and their fate and effect mechanisms must be identified to protect the integrity of ecosystem and human health. The first problem with these compounds is that their concentrations in receiving waters are too low to detect through wet analysis. Since the information on their occurrence is the starting point for the evaluation of their fate and effect in the environment, a rapid and sensitive method was developed to measure the concentrations of 14 pharmaceuticals and hormones in surface water. Good peak shapes and chromatographic separation preventing cross-talks in MS/MS were achieved with application of ultra-performance liquid chromatography. The 1.9 μm-particulate size-column enabled low run times and consequently decreased solvent consumption. With the developed method, not only low detection limits (0.1-1 ng/L depending on the compound) were achieved but also it is possible to use it to measure compounds having a wide range of concentrations from ng/L to μg/L levels. To sum up, the developed method is one of the few in the literature for multi-residue analysis of both pharmaceuticals and hormones.

The method developed during the study was used in order to monitor the presence of pharmaceuticals and hormones in a drinking water source: Büyükçekmece Lake and its main tributaries (Karasu, Hamza, and Tahtaköprü Rivers; Ahlat and Beylikçayı Creeks). Concentrations and detection frequencies for all/almost all compounds were lower in Büyükçekmece Lake than in its tributaries. The low concentrations in Büyükçekmece Lake can be explained by the high volume and retention time of water in the lake compared to the rivers. Among the rivers and creeks, Ahlat and Beylikçayı Creeks had the highest pharmaceutical concentrations. The

concentrations of pharmaceuticals were so high that even though they are small creeks with rather low flowrates, their contribution to the pollution load to the lake is not negligible.

Most of the pharmaceuticals were detected in high frequencies in rivers and creeks with antibiotics and caffeine being the most frequently detected pharmaceuticals. The concentrations of pharmaceuticals were different at several orders of magnitude with some pharmaceuticals having concentrations below 10 ng/L and some having concentrations of 10 µg/L. Even pharmaceuticals of which only 1% is excreted as unchanged compound have been detected in some samples. Propranolol is an example of such compounds and propranolol's adverse effects to aquatic species at low concentrations also suggest the importance of occurrence studies with proper analytical techniques.

There is no wastewater treatment plant discharging treated wastewater into the upstream of the sampling points. Therefore, the presence of such high concentrations suggests that there are some uncontrolled wastewater discharges to the rivers and creeks. Therefore, the results of this study indicate that immediate measures should be taken for unknown or uncontrolled wastewater discharges in Büyükçekmece Watershed.

The occurrence studies also shed some light on the persistency of pharmaceuticals. The measurement results confirm that amoxicillin is prone to degradation in environment via natural degradation processes. On the other hand, ciprofloxacin and erythromycin are persistent to degradation. Considering the persistency of some compounds such as ciprofloxacin, continuous loading of pharmaceuticals through the creeks may lead to accumulation in the lake and hence may threat the human health in addition to the ecosystem, since Büyükçekmece Lake water is used to supply drinking water to approximately 2 million people in Istanbul.

The detection frequency of hormones was lower than the pharmaceuticals. Among the hormones, E3 was detected more and had higher concentrations compared to E1 and E2. These results support the theory about the conversion of hormones from one to other and that E1 and E2 are converted to E3 by natural processes.

There are fluctuations in concentrations of target compounds from season to season. Highest concentrations were observed particularly during July sampling period

corresponding to dry weather conditions. Since seasons have a significant effect on the concentration of pharmaceuticals, the need for sampling throughout the year to capture any seasonal effect is underlined. Moreover, this study indicates that although the same environmental concentrations are expected within a community based on the pharmaceutical and water usage rates, data obtained from different sampling points in the watershed may differ significantly.

In addition to occurrence studies, the possible effects of pharmaceuticals and hormones have also been studied. *D. magna* acute immobilization test, *P. subcapitata* growth inhibition test, *D. magna* reproduction inhibition test, AMES test, and YES test were conducted to achieve information about acute, chronic, mutagenic, and estrogenic effects of pharmaceuticals and hormones.

Pharmaceuticals and hormones affect *D. magna* acutely at mg/L concentration levels which is unlikely to observe in environmental waters. On the other hand, *P. subcapitata* was more sensitive than *D. magna* to pharmaceuticals and hormones. Although several compounds such as atenolol, ibuprofen, and caffeine have higher EC50 values for *P. subcapitata* growth inhibition test, their NOECs for *P. subcapitata* growth inhibition test are much lower than *D. magna* acute immobilization test except atenolol. These results indicate the importance of conducting ecotoxicological studies with various species at different levels of the food chain. Since only one effect is not enough to compare and classify ecotoxicological analysis, chronic tests were conducted in addition to the acute toxicity tests. *D. magna* reproduction inhibition test endpoints were at µg/L level for all compounds except atenolol for which the endpoint was at mg/L level. The differences observed between acute and chronic effects of pharmaceuticals and hormones on *P. subcapitata* and *D. magna* indicate that even tough studied pharmaceuticals and hormones may not present acute adverse effects at low concentrations; they may have drastic chronic effects.

Although sulfamethoxazole was the most ecotoxic antibiotic according to *D. magna* acute immobilization test, erythromycin was the most ecotoxic antibiotic according to *P. subcapitata* growth inhibition and *D. magna* growth inhibition tests. On the other hand, amoxicillin induced endocrine disruption at lowest concentration (5 µg/L) among antibiotics. However, ciprofloxacin had highest estrogenic potential with 28±3% RIE among all tested compounds.

Even though *P. subcapitata* do not have β-receptors suggesting that they may not be affected at all by the β-blocker, the effect of propranolol on *P. subcapitata* was more than the acute effect on *D. magna.* Therefore, it is possible that there is an additional effect mechanism of propranolol other than blocking the β-receptors. However, dissimilar results were observed for atenolol where *D. magna* is affected more than *P. subcapitata*. On the other hand, atenolol started to exert an estrogenic effect at 500 ng/L which is the lowest concentration among all tested pharmaceuticals.

The hormones studied did not trigger any acute effect to *D. magna* at bioavailable concentrations. However, they have growth inhibition effect to *P. subcapitata* and reproduction inhibition effect to *D. magna*. Although their effects were approximately 100 times higher for *D. magna* chronic toxicity test compared to *P. subcapitata* growth inhibition test at low concentrations, all compounds had similar effects to *P. subcapitata* and to the reproduction of *D. magna* at higher concentrations. EE2 was the only compound that had any mutagenic effect.

Among tested NSAIDs, while ibuprofen was the most ecotoxic compound at higher concentrations, at lower concentrations naproxen was the most ecotoxic compound. This is valid even for different ecotoxicity tests. Ibuprofen was the most ecotoxic NSAID for *D. magna* acute immobilization and for *P. subcapitata* growth inhibition tests until 1 mg/L concentration. Naproxen was the most ecotoxic compound for *P. subcapitata* growth inhibition test and *D. magna* reproduction inhibition test below 1 mg/L. The estrogenic effect of all NSAIDs starts after 50 µg/L and they have rather lower RIE except ibuprofen with 20±3% RIE.

No ecotoxic effect is expected for concentrations obtained in samples taken in February, March, May and October due to low concentrations of pharmaceuticals and hormones in those samples. On the other hand, concentrations in July are high enough to exhibit ecotoxicological effect. In particular, extremely high ciprofloxacin and naproxen concentrations may cause chronic effects on several species.

Since pharmaceuticals and hormones present in the aquatic environment with other pharmaceuticals and hormones, their mixture effects should be identified to get information on interactions among these chemicals. For this purpose, mixtures of therapeutic groups and mixtures of all compounds ecotoxicologically tested.

Tests conducted with therapeutic group mixtures showed the most interesting results. All mixtures had synergistic interaction for *D. magna* acute immobilization, *P. subcapitata* growth inhibition, and *D. magna* reproduction inhibition tests. Moreover, mixtures had stronger toxicity than predicted values even at which single compounds do not exhibit effects for *D. magna* acute immobilization, *P. subcapitata* growth inhibition, and *D. magna* reproduction inhibition tests. These results indicate that NOECs for single toxicity tests are not enough for assessment of environmental risks of the compounds. Hormone mixtures also caused mutagenic effects due to mutagenicity of EE2. Based on the mixture results synergistic interaction of amoxicillin, ciprofloxacin, and naproxen is expected in Ahlat in June since the concentrations measured are higher than the concentrations used in the mixture to determine chronic effects of total mixture.

For YES test, neither interaction of compounds nor additive effects were observed. Estrogenic activity of the mixtures was not statistically different from the compound of which has highest estrogenic activity in the mixture which means that highest estrogenic compound dominates estrogenicity of mixtures. Since in all sampling periods, Büyükçekmece Lake and some of its tributaries contain hormones, it can be concluded that all of the samples in all sampling periods (except Beylikçayı in October and Ahlat in July) will exhibit estrogenic effect.

The occurrence and ecotoxicological data obtained during this study are important for environmental risk assessment of pharmaceuticals and hormones. Particularly, the results of mixture tests provide valuable information to risk analysts and decision makers as well as to the scientific literature. Particularly, there is no study in the literature reporting *D. magna* 21d reproduction inhibition test of pharmaceutical and hormones and the results obtained in this study will be the first.

Future works should focus on identifying interactions among more pharmaceuticals and endocrine disrupting compounds. More ecotoxicological tools with higher species (*e.g.,* vitellogenin synthesis in male fish and inhibition to embryonic development of fish) or macrocosms should be used to identify effects through food web and species interactions since it is not possible to extrapolate ecotoxicological data from one species to another one.

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Professional Experience and Rewards:

2004 - : Research assistant at Istanbul Technical University, Environmental Engineering Department

September 2007 – December 2007: Guest researcher at University of Naples "Federico II"

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Tinçel Culture Foundation International Research Fellowship (3 months)

TÜBİTAK – 2214 International Research Fellowship for PhD. students (9 months)

List of Publications and Patents:

SCI Journal Articles:

Aydin, E., Yaman, F. B., Ates-Genceli, E., Topuz, E., Erdim, E., Gurel, M., Ipek, M., Pehlivanoglu-Mantas, E. 2012. Occurrence of THM and NDMA precursors in a watershed: Effect of seasons and anthropogenic pollution. *Journal of Hazardous Materials*. 221-222, 86-91.

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Engineering Index Journal Articles

Lofrano, G., **Aydin, E**., Russo, F., Guida, M., Belgiorno, V., Meric, S. 2008. Characterization, Fluxes and Toxicity of Leather Tanning Bath Chemicals in a Large Tanning District Area (IT), *Water Air Soil Pollut: Focus*, 8(5-6), 529-542

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Talinli, I., Topuz, E., **Aydin, E**., Kabakci, S. (2011). A Holistic Approach for Wind Farm Site Selection by Using FAHP, Wind Farm - Technical Regulations, Potential Estimation and Siting Assessment, Gastón O. Suvire (Ed.), ISBN: 978-953-307-483-2, InTech

International Conference Presentations:

Talinli, I., **Aydin, E**., Yamanturk, R. 2005. Determination of Hazardous Wastes from Foundry: Toxic Criterion with BioTox Toxicity Test, 13. *International Symposium of MESAEP*, Thessalonica, Greece

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Pehlivanoglu Mantas, E., **Aydin, E**., Insel, G., Ozgun, H., Cakmakci, M., Ozturk, D. C., Ubay Cokgor, E., Ozturk, I. 2007. Conceptual Approach for the Determination of Point Sources in Melen Watershed. *International River Basin Management Congress*. 22-24 Mart 2007, Antalya, Turkey

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