A study of the distribution of selected anthropogenic micropollutants in the seawater of Dublin bay

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by

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Declaration

I certify that this material, which I now submit for assessment on the programme of study leading to the award of M.Sc., is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text,

Signed: Francis J Roden Date: 24/07/07

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Contents

Abstract

Since the middle of the last century there has been a growing awareness of the existence of widespread environmental pollution and of the potentially harmful effects that polluting species can have on biological systems.

Initially the concern focused on the ubiquity and on the harmful effects of organochlorine compounds, in particular organochlorine pesticides.

In the 1970s the malign effect that xeno-estrogenic material could have on biological systems became apparent and the increasing incidence of such polluting material, frequently derived from sewage or other wastewater effluent, in the aquatic environment provoked concern.

A more recent topic for research has been the occurrence in the aquatic environment of the residues of pharmaceuticals originally developed and prescribed for the treatment of human or animal illnesses or physiological complaints. Though ample evidence has been recorded for the prevalence of such residues no definite conclusions have been reached regarding the effects of such pollution.

This work targeted six compounds that were deemed to have potential for anthropogenic pollution. The species selected were caffeine, methylparaben, estradiol, ethynylestradiol, ibuprofen and nonylphenol. Dublin bay was chosen as a sampling site.

HPLC methods for the identification and the quantification of each of these compounds were developed. The method for the detection of caffeine had a limit of detection (LOD) of 0.005 mgL^{-1} and a limit of quantification (LOQ) of 0.02 mgL^{-1}. The method for estradiol, which utilised the natural fluorescence of this compound as a means of detection, had a LOD of 0.0025 mgL^{-1} and a LOQ of 0.05 mgL^{-1}.

A solid phase extraction method for the extraction of these compounds from seawater was established. This method had a recovery rate of 83.2% for caffeine and 88.9% for estradiol.

Items of glassware that would allow the simultaneous preparation of up to 12 extracts of seawater that would be suitable for HPLC analysis were designed.

Chapter 1

Introduction

Environmental pollution, the background to solid phase extraction and sampling procedures

1.1 Environmental pollution

1.1.1: The development of the phenomenon of widespread environmental pollution

It has, probably, always been realized that excessive discharge of the refuse of human living into rivers or lakes has the potential to affect detrimentally the state of those waterways and that such deterioration in the quality of water sources inevitably has deleterious consequences for local communities. In contrast, the oceans have until recently, been seen as sinks of almost infinite capacity for such refuse and the divertion of unwanted materials to the sea has been viewed as a convenience that would have negligible, if any, consequences for mankind.

Though archaeology has revealed instances where prehistoric copper refining did cause extensive pollution of both marine and coastal habitats [1], this view was, largely, justifiable since the bulk of such refuse was of an organic nature and hence biodegradable. However, with the development of the chemical industry in the 1930s a range of non-biodegradable chemicals became available and the implicit acceptance of this view could no longer remain unchallenged.

After the Second World War the range of such chemicals and the quantities that were being produced increased dramatically. That such developments were not environmentally neutral and that widespread contamination of the environment was possible were brought to public awareness by several occurrences.

The contamination of fish by organic mercury that had been discharged into the sea at Minamata, Japan, in the 1950s gave rise to a disease in the local population that used these fish as part of their diet. More than 900 people died as a consequence of eating locally caught fish and thousands were left permanently disabled as the disease attacked their nervous system causing blindness, seizures, many sensory disorders and malformed pregnancies.

In the early 1960s the decline in the population of certain birds of prey was observed, e.g. the bald eagle in North America and the white tailed eagle in the Baltic. This was attributed to the widespread use of the insecticide, DDT.

In 1968 in Yusho, Japan, a brand of rice oil contaminated with polychlorinated biphenyls was made available. About 1800 people who had ingested this product subsequently presented with severe clinical symptoms.

As an example of a synthetic material that was initially perceived to be of benefit to mankind but subsequently found to have disadvantages, it is, perhaps, worth recalling some of the circumstances surounding the use of DDT (Dichloro-diphenyl-trichloroethane). This compound was first synthesised in 1874 by the German chemist, Othmar Zeidler. In 1939 a Swiss chemist, Paul Mueller, recognised its effective insecticidal properties and for this work he was awarded the Nobel prize in medicine in 1948. Prior to this observation pest control in horticulture was achieved with such toxic compounds as lead or calcium arsenate. DDT was used extensively during the 1950s and 1960s to control mosquitoes and agricultural pests. The detrimental effects that DDT was having on wild life were gradually recognised during the late 1950s . These effects ranged from the observation of dead birds in fields that had been sprayed with the compound to the persistance of oviducts in male birds that otherwise appeared quite healthy. Shell thinning and consequent reduction in numbers was a common observation in eagles and other birds of prey- i.e. top predators.

In the late 1950s observations such as these prompted widespread concern amongst scientists and others. In 1962 these concerns were highlighted by an employee of the US Fish and Wildlife service, Rachel Carson, in her book entitled, "Silent Spring" [2]. In 1970 legislation restricted the use of DDT and shortly thereafter its use was eliminated in the USA.

That DDT had the potential to be profoundly disruptive to the normal development of wild-life might have been guessed from implications of work that had been carried out shortly after its introduction as an insecticide. In the early 1950s Burlington [3] showed that it could have the effect of feminising cockerels. However this warning, apparently, went unnoticed.

1.1.2: Conventions for monitoring the environment [4]

These and other incidents prompted governments and public bodies to establish environmental monitoring groups that might study the status of the marine environment and attempt to detect trends in the concentration of contaminants. There are at present many such groups; the following are considered to be relevant to the Irish situation.

The Stockholm convention (2001)

This is a treaty that has been signed by 151 countries including Ireland. The aim is to protect human health and the environment from persistent organic pollutants (POP). Signatories to this convention undertake to reduce or to eliminate the release of selected POPs. The species selected are the twelve chemicals previously selected by the United Nations Environment Programme. They are, aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs) and toxaphene.

Convention for the Protection of the Marine Environment of the North East Atlantic (OSPAR)

This group was formed in 1992 by the merger of two previous commissions, The Oslo commission and the Paris Commission. Its objective is "the cessation of discharges, emissions and losses of hazardous substances by 2020 with the aim of achieving concentrations in the marine environment close to background levels for naturally occurring substances and close to zero for man-made synthetic substances". The list of priority pollutants specified by OSPAR is lengthy, however, they can be grouped with some representative species from each group (cf. Table 1.1)

The European Commission and the Water Framework Directive (WFD):

This directive was proposed in 1997 and adopted by the Parliament and Council in 2000 (2000/60/EC). It requires all member states to monitor the concentration of selected pollutants in groundwaters and coastal waters. The methods of

sampling and of analysis are specified so that comparison between different regions is possible.

The Environmental Protection Agency (EPA, Ireland)

The EPA was established in 1993 by the Environmental Protection Agency Act of 1992. It has a wide range of statutory duties and powers. Amongst these are responsibility for implementing the monitoring requirements of transitional, coastal and marine waters required by the WFD (2000/60/EC) and OSPAR

Table 1.1: Some priority pollutants specified by OSPAR.

Group	Example
"Old" Organochlorines	Hexachlorocyclohexane Isomers
	Toxaphene
	Endosulphan
"New" Pesticides	Methoxychlor
	Pentachlorophenol
Volatile Organic compounds (VOCs)	1,2,3-Trichlorobenzene
Polyaromatic Hydrocarbons (PAHs)	Anthracene
"New" Organohalogens	Hexachlorobutadiene
	Tetrabromobisphenol-A (TBBP-A)
Endocrine Disruptors	Nonylphenol
	Diethylhexylphthalate (DEHP)
Other Organic Chemicals	Clotrimazole
	Musk Xylene
Metals	Mercury and Organic mercury compounds
	Organic Tin compounds

1.1.3: Research into the distribution of particular OSPAR priority pollutants (Organochlorines and polyaromatic hydrocarbons)

The ubiquity of organochlorine pesticides is demonstrated by numerous reports. These include the finding of toxaphene in the tissue of Norwegian whales [5] and of DDT in that of two species of fish sampled in a Portugese estuary [6]. Such compounds have also been shown to be present in seawater samples taken at Hong Kong [7], Singapore [8, 9], the Mediteranean [10], the Baltic Sea [11], the Irish Sea [12] and in the river systems of Holland [13]. In this latter report DDT was not detected though simazine and atrazine, which are on the WFD list of persistent organic pollutants (POPs), were detected. These compounds are not on the OSPAR list.

The reported concentrations of organochlorines in corresponding matrices from different regions vary considerably. Table 1.2 lists some representative figures.

Table 1.2: Organochlorine pesticides in different matrices

Organochlorine Pesticide and Matrix.	Concentration	Reference
Toxaphene in Norwegian Whales.	11,447 ngg^{-1} l.w.	5
DDT in Seawater (Singapore)	0.1 ngL^{-1*}	9
DDT in Seawater (Hong Kong)	1.908 ngL^{-1}	7
DDT in flounder liver (Portugal)	301 ngg^{-1} d.w.	6
DDT in Baltic Seawater	0.14 ngL^{-1}	11
Simazine in Dutch rivers.	80 ngL^{-1+}	13
Atrazine in Dutch rivers.	200 ngL^{-1+}	13

* Samples taken 1m below the surface.
+ Several rivers were sampled; these are the maximum values measured.

1.1.4: Pharmaceutical residues in the aquatic environment

An impressive feature of modern medical and of veterinary science is the range of drugs that it can offer to practitioners who are confronted with the illnesses and the conditions that afflict mankind and domesticated animals. Approximately 3000 pharmaceuticals are registered for use in the UK [14]. Some of these have been extracted from natural sources but many have been synthesised to achieve a specific biochemical effect. They include antibiotics, analgesics, antipyretics, anti-depressants, antihistamines, formulations to control hypertension, neuralgia and many other materials designed to alleviate specific health problems.

Though it was probably not forseen, it was inevitable, that the residues of such materials would, sooner or later, find their way into ground and surface water.

In the early 1980s the attention of research workers was drawn to the fate of the residues of such pharmaceutical compounds. Since that time this topic has become an area of intense research activity. Pharmaceuticals have been found both in the effluent from Sewage Treatment Works (STP) [15,14] and in the receiving waters of that effluent both above and below the point of discharge [16,17], in harbours [18] and in estuaries [19,20] and in the open sea [21,19]. Research of this nature was initially carried out in Europe [22], but it has been repeated in the USA [23,24], in Australia [15] and in the Middle East [25].

The very large quantities of such compounds that are distributed annually suggests that pharmaceutical residues might be present in the environment in detectable concentrations (cf. Table 1.3). They are often lipophilic and persistent, the very properties that should allow them to bioaccumulate. They have been synthesised to achieve a specific physiological effect so they are highly likely to provoke biochemical effects in the habitats to which they are jettisoned.

Table 1.3: Annual consumption of certain prescribed drugs (t/year) [26]

	Germany (2001)	Austria (1997)	England (2000)	Switzerland (2004)
Paracetamol	621.65	35.08	390.9	95.2
Ibuprofen	344.89	6.7	162.2	25.0
Diclofenac	85.8	6.14	26.12	4.5
Carbamazepine	87.6	6.33	40.35	4.40

Though the effects of the "old" organochlorines (e.g. DDT) and of endocrine disrupting chemicals (EDC) (see below) have been well established, reviewers frequently refer to the lack of knowledge that exists about the effect that pharmaceutical residues may have on the environment. By their nature such effects are likely to be unforeseen, e.g. the recently observed high death rate amongst three species of raptor in India and Pakistan has been attributed to diclofenac. This drug is used extensively on domestic cattle in these countries. The birds in question prey on any dead carcase and the drug accumulates within their bodies provoking renal failure and eventual death [26].

Included in this category of pollutants are all drugs and medications that are used to ameliorate the ailments of man or of animals. When used for the former purpose they will be excreted unchanged or in a metabolised or conjugated form and find their way to a Sewage Treatment Plant (STP). Halling-Sorensen *et al.* have drawn attention to the fact that "an unknown portion of marketed human medicinal substances ends in the sewer system as surplus medical substances considered as waste" [27].

Prior to leaving the body pharmaceuticals may undergo what has been described as Phase 1 and/or Phase 2 reactions.

Phase 1- the pharmaceutical undergoes oxidation, reduction or hydrolysis. The products of this reaction are often more reactive and sometimes more toxic than the parent compound.

Phase 2- this involves conjugation with another molecule resulting in a complex that is usually physiologically inactive.

1.1.5: Fate of a pharmaceutical within a sewage treatment plant (STP)

In a neutral, aqueous environment (pH 7), acidic pharmaceuticals i.e. those with a low pK_a (e.g. Ibuprofen), will be almost totally deprotonated. As charged species they will tend to be dissolved rather than adsorbed to any hydrophobic particulate material, i.e. sludge. At a very low pH the opposite will be the case.

The products of Phase 1 and Phase 2 reactions are invariably more water soluble than the parent compound, a transformation that helps ensure their excretion from the body in the urine. Frequently microbial action releases the free drug from its conjugated form when the metabolite reaches the aquatic environment [27].

The products of a STP are sludge and treated sewage water, both of which may contain pharmaceutical residues. The sludge may be dumped at sea or, after appropriate sterilization, be spread on land. In the latter case any contained pharmaceutical residues are liable to be leached into the aquatic environment. The treated sewage water is returned to rivers or to the sea.

Veterinary medical compounds are used not only for therapeutic purposes but also as growth promoters. In either case they or their metabolites or conjugated forms are excreted. The excrement in which such species are incorporated is invariably spread, either immediately or after storage, on fields. Hence any contained pharmaceutical residues are liable to be leached into the surrounding waterways.

1.1.6: Concentrations of pharmaceuticals in the aquatic environment

Though there are approximately 3000 human pharmaceutical substances registered for use in the UK, research into pharmaceutical residues in the environment has tended to focus on a small number of such substances. The UK Environmental Agency has identified 13 such compounds for priority investigation: these are, acetylsulfamethoxazole, clofibric acid, dextropropoxyphene, diclofenac, erythromycin, ibuprofen, lofepramine, mefenamic acid, paracetamol, propranolol, sulfamethoxazole, tamoxifen, trimethoprim.

Apart from these there are reports on the distribution of caffeine, carbamazepine and the synthetic estrogen, ethynylestradiol. Reports on the concentrations of pharmaceuticals in the aqueous environment vary considerably. Tables 1.4, 1.5 and 1.6 show representative values.

Table1.4: Concentrations of pharmaceuticals in sewage effluent (ngL^{-1})

Pharmaceutical			
Mefenamic Acid	1100 [14]		273 [16]
Diclofenac	460 [14]	990 [17]	599 [16]
Propranolol	180 [14]		93 [16]
Clofibric Acid	< 50 [14]	60 [17]	
Ibuprofen	1800 [14]	1300 [17]	4201 [16]
Carbamazepine		950 [17]	
Naproxen		2600 [17]	
Ethynylestradiol	2.6 [41]		
Caffeine	1000 [29]		

Table 1.5: Concentration of pharmaceuticals in rivers and streams (ngL^{-1})

Pharmaceutical			
Mefenamic Acid			
Diclofenac		33 [30]	< 20 [16]*
Propranolol			115 [16]*
Clofibric Acid		4.7 [30]	
Ibuprofen	1000 [23]	11 [30]	1555 [16]*
Carbamazepine	30 [13]		
Naproxen			
Ethynylestradiol	0.831 [23]	2.5 [25]	(1.8 -2.5) [25]
Caffeine	6000 [23]	104 [30]	(98 – 176) [30]

* This research includes data for sewage effluent and for the river water above and below the point of discharge of the effluent. These figures refer to the maximum values found in the streams above the point of discharge.

Table1.6: Concentration of pharmaceuticals in the open sea and in estuaries, (ngL^{-1})

Pharmaceutical	Open sea	Belfast Lough	A Norwegian harbour
Mefenamic Acid		196 [20]	
Diclofenac		< 8 [20]	
Propranolol		45 [20]	
Clofibric Acid	1.35 [19]+	111 [20]	
Ibuprofen		124 [20]	0.3 [18]
Carbamazepine			
N,N-diethyl-3-toluamide++	1.09 [19]+		10 [18]
Caffeine	16.1 [19]+		87 [18]

+ This research was carried out in different locations in the North Sea. These figures are the maximum values obtained.

++ This is a commonly used insect repellent.

1.1.7: The phenomenon of endocrine disruption

The term endocrine disrupting chemical (EDC) was popularised, if not coined, by the authors of "Our Stolen Future" [31] first published in 1996. This is a popular and somewhat zealous account of the detrimental effects which several synthetic chemicals had had on the health and the well-being of man and of animals since they were first used extensively about forty years previously.

When they were first introduced these chemicals had been perceived to have major economic or therapeutic advantages. Subsequently it was discovered that they could have insidious and profoundly deleterious effects on wild-life and on human health and well-being. The unfortunate physiological effects were achieved by an interruption of the normal pre-natal development of the organism, a process that is under both genetic and hormonal control. The malign interruptive effect is achieved when an exogenous chemical rather than the appropriate hormone forms a bond with the hormone receptor.

Perhaps the classic case of an EDC is that of the compound diethylstilbestrol (DES). This compound was first synthesised in 1938 by Edward Charles Dodd. It was found to have physiological properties comparable to natural estrogens and for a period of almost 30 years members of the medical profession promoted its use in the treatment of problematic pregnancies, believing that the difficulties were caused by estrogen deficiency. It was also used to treat other conditions perceived to to be caused by hormonal imbalance, e.g. amenorrhoea and for the suppression of lactation [32]. Prior to 1976 DES was added to the feed of chickens and of cattle since it significantly increases the efficiency with which feed is converted to meat. This practice is no longer legal.

The deleterious effects of DES became evident when daughters of women who had been treated with the drug reached their teens and early twenties. A significant incidence of malformed reproductive tracts and reproductive tract cancers were observed in these young women, even though their mothers had shown no adverse reactions to the drug.

When these disorders were first observed there was a reluctance amongst the medical profession to accept that a drug administered to a pregnant woman could cause a disorder or even a cancer in the child fifteen or twenty years later. This reluctance was a consequence of the belief that the placenta acted as a barrier, protecting the unborn child from any harmful substance that might be present in the mother's bloodstream. This belief, firmly held for many years, was to be undermined by the consequences of the administration of thalidomide in the early 1960s and of DES a decade later.

Though the detrimental consequences of using DES as a prescription drug were incontrovertible, the human health implications of minute concentrations of estrogenic compounds in the environment is less clear cut. Nevertheless there are a variety of human health issues for which such EDCs have been proposed as, at least, a probable cause. These include reduction in sperm count, increased incidence of testicular and prostate cancer, increased incidence of cryptorchidism* [33] and hypospadias+, altered sex ratio, increased incidence of female breast cancer and various neurological effects [34].

1.1.8: The effects of EDCs on lower mammals, on fish, reptiles and invertebrates

Following an accidental discharge of the chemical difocol into Lake Apokpo, Florida, in the early 1980s alligators failed to breed successfully in the lake even though all apparent evidence of the spill had been removed. This failure was found to be due to malformed reproductive organs, a morphological response presumed to have been provoked by trace levels of the pollutant remaining in the water [35]. In the 1990s abnormal sexual development of fish from U.K. rivers was shown to be associated with the presence of sewage effluent in those rivers [36]. There are

* Cryptorchidism: a condition in which one or both of the testes fail to descend from the abdomen into the scrotum.

+ Hypospadias: condition in males in which the opening of the urethra is on the underside of the penis.

frequent reports that exposure to EDCs provokes the development of the female specific protein vitellogenin, in male fish [37, 38], in fact this established response is used as a test for the presence of such species in waterways.

The estrogenic properties of 4-nonylphenol were discovered serendipitously by Soto and Sonnenschein in 1987 at the Tufts Medical School in Boston. Their experiments involved breast cancer cells. The proliferation of these cells is normally kept under control by an inhibiting substance in the blood. An effect of estradiol is to over-ride this inhibitor and to permit the proliferation of these cells. In the absence of estradiol they noticed that proliferation of these cells was promoted by a substance that leached from the polystyrene vessels that contained the cell cultures. The leachate was identified as 4-nonylphenol. Subsequently they showed that this compound would cause endometriosis in rats, a condition that can also be provoked by estradiol.

Some years later in a different laboratory a chance discovery somewhat similar to that of Soto and Sonnenchein showed that bisphenol-A (BPA) has estrogenic properties [35]. In this case the contaminating BPA was polycarbonate laboratory vessels. The ubiquitous nature of BPA can be judged from the following, "Used in the manufacture of polycarbonate plastics and epoxy resins, BPA is present in a multitude of products including the interior coating of food cans, wine storage vats, water carboys, milk containers, food storage vessels, baby formula bottles, water pipes, dental materials, automotive lenses, optical lenses, protective coatings, adhesives, protective window glazing, compact discs, thermal paper, paper coatings, and as a developer in dyes" [35].

Markey *et al.* [35] have summarised the effects that BPA can provoke in mice. These include malformed reproductive tracts in males and in females, malformed mammary glands in females and altered sociosexual behaviour in both males and females. It has also been suggested that low level exposure to estrogenic

substances during the smolt* runs to sea may be one of a number of factors responsible for the decline in catches of Atlantic salmon [39].

1.1.9: The prevalence of endocrine disrupting chemicals (EDCs)

Throughout the last two decades there have been frequent reports from many different countries on the presence of EDCs in a wide variety of environmental waters. The chemicals in question have included alkylphenols, alkylphenol ethoxylates, natural and synthetic estrogens, bisphenol-A (BPA), and phthalates. The following results are presented to demonstrate the ubiquity of such species (Table 1.7).

* Smolt – A young salmon after the parr stage, when it becomes silvery and migrates to the sea for the first time.

Table 1.7: Examples of EDCs in environmental waters

Matrix/Country	Species	Concentration	Reference
River Water downstream of WWTP*/Spain	4-Nonylphenol	355 μgL^{-1}	38
Seawater/Baltic Sea	4-Nonylphenol BPA Ethynylestradiol Estrone	(2.5 – 13.8) ngL^{-1} (0.22 -5.4) ngL^{-1} (< 0.45 - 17.2)ngL^{-1} (0.10 -0.53) ngL^{-1}	40
STP effluent (The Netherlands)	4-Nonylphenol BPA Ethynylestradiol Estrone	(550 -1500) ngL^{-1} (43 - 4090) ngL^{-1} (< 0.3 -2,6) ngL^{-1} (< 0.3 – 11) ngL^{-1}	41
River Sediment/Spain	Diethylstilbestrol Ethynylestradiol Estrone	(n.d. -2.01) ngg^{-1} (n.d. – 22.8) ngg^{-1} (n.d. -11.9) ngg^{-1}	42
Sludge from industrial WWTP	4-Nonylphenol	5.13 μgg^{-1}	43
Clarified water from STP (Canada)	4-Nonylphenol	2.900μgL^{-1}	44
Suspended solids from STP (Canada)	4-Nonylphenol	73μgg^{-1}	44
Rainwater (Netherlands)	Diethylphthalate BPA Di(2- ethylhexyl phthalate)	(240 – 340) ngL^{-1} (< 15 – 57) ngL^{-1} (690 – 1700) ngL^{-1}	41

* WWTP Waste water treatment plant.

1.1.10: Anthropogenic molecular markers and the concept of biomarkers

Anthropogenic molecular markers are substances that are indicative of human presence and/or human activity. Three categories of such materials can be distinguished:

1. Uniquely human metabolic products, e.g. 5β-coprostanol.
2. Synthetic compounds that are not encountered in nature, e.g. 4-nonylphenol.
3. Natural occurring materials that are not encountered in the regions in which they are to be used as markers other than by anthropogenic transport, e.g. caffeine.

To be useful as a molecular marker a compound must be persistent in the environment or it must, at least maintain sufficient structural integrity for its source to be recognised [45].

Materials that have been used for this purpose include 5β-coprostanol, or a combination of this compound and trialkylamines, both of which occur in sewage [46]. Though coprostanol occurs in the excreta of all vertebrates, the sterols in human faeces show distribution profiles considerably different from those in other animal excreta [45, 47, 48].

Linear alkyl benzenes, the degradation products of domestic detergents, and trialkylamines have also been used to trace sewage effluent [49]. Lignin, a phenolic polymer that constitutes the structural material of plants, has been used to follow the terrestrial input to marine waterways [50]. Carbamazepine, an anti-epileptic drug, seems to be very persistent in the environment and therefore qualifies, in this respect, as a possible marker [51]. Pristane (2,6,10,14-

tetramethylpentadecane) has been detected in marine sediment in the vicinity of fish farms and can be used to follow the dispersal of organic waste from such sites [52]. Stilbene-type fluorescent whitening agents that are found in laundry detergents, have been used to follow the transport of sediment partcles in Tokyo Bay [53].

In some instances a background concentration of a particular pollutant may exist and superimposed on this localised peak concentrations may occur. e.g. Ricking *et al.* [54] measured a background concentration of 1500-2500 ng/g of the PVC plasticisers, alkylsulfonic acid esters, in the sediment of a German river system. This value peaked at 8860 ng/g enabling them to identify a distinct emission source. In this river system which receives a high input of anthropogenic contaminants, a range of anthropogenic marker compounds has been suggested. This range includes, detergent related substances, plasticizers, fragrances and UV-protectors.

An obvious group of compounds to consider in this regard is the hydrocarbons. This will include not only petroleum related substances but also compounds formed by the microbial and chemical degradation of naturally occurring lipids.

Petroleum related hydrocarbons enter the environment through shipping activities, sewage disposal, offshore oil production and transport, oil spills and pyrolysis and combustion of fossil fuels. The mixtures of hydrocarbons that are encountered in environmental research frequently contain components of both biogenic and of anthropogenic origin. Careful discrimination is required to assign the different constituents to their appropriate source or origin. Such work has been carried out, e.g. in Patos Lagoon, Brazil [55].

The concept of biomarkers

The simple measurement of a contaminating species in environmental waters by chemical means conveys no information about the effect of that contaminant on the environmental flora or fauna. Additionally the concentration of that species may be extremely low and therefore difficult to measure with any great accuracy. An approach that has been used to cope with these difficulties is that of the

biomarker. This is a cellular component of an organism that is sensitive to the contaminant, often in very low concentration, and that can provide immediate information on the effect of the contaminant.

Acetylcholinesterase in mussels and in red mullet has been used for such studies in the seas surrounding the Salento Peninsula of Southern Italy [56] and in France copepods* have been used for the same purpose [57].

Acetylcholinesterase is an enzyme that breaks down the neurotransmitter, acetylcholine, at the synaptic cleft (the space between two nerve cells) so that the next nerve impulse can be transported across the synaptic gap. Pesticides of the organophosphate and carbamate types act to kill or to paralyse insects by inhibiting their acetylcholinesterase.

Bivalved molluscs have been used for a similar purpose in Antartica [58].

The use of the three-spined stickleback fish (*Gasterosteus aculeatus* L), which responds to androgens in the environment by producing within its kidney a unique protein called spiggin, falls into this category [59] as does the production of vitelogenin in male fish in response to EDCs [57, 38], and the decrease in phenoloxidase activity in the estuarine tunicate, *Styela plicata,* in response to TBT (tributyltin) contamination [60].

* A copepod is an aquatic crustacean with paddle like feet.

1.2: Solid phase extraction

1.2.1: Extraction and preconcentration of micropollutants

The analyst who seeks to identify and to quantify micropollutants in environmental samples is inevitably confronted with the problems that are inherent in the low concentrations of such compounds and in the complexity of the matrices in which they occur. The concentrations of such species are such that low ngL^{-1} are routinely encountered and frequently pgL^{-1} are considered to be significant. In its passage over soil and rocks, surface water will inevitably come in contact with a variety of species, of both organic and inorganic origin. Owing to the effective solvating power of water many of these will be present, to a greater or lesser extent, as solutes, in the aqueous sample. Thus, prior to the identification and to the quantification of the analytes of interest, their extraction and their concentration are essential.

Traditionally extraction and concentration was by means of liquid/liquid extraction (LLE). This is achieved by placing a specific volume of the aqueous sample in a separating funnel together with a volume of a liquid that is both immiscible with water and that is comparatively more volatile. The two liquids are then shaken and subsequently allowed to separate. By carefully choosing the organic liquid the analyte will be distributed between the liquids in such a way that at equilibrium there will be a greater amount of the analyte in the organic than in the aqueous phase. The aqueous and the organic phases are then separated and the procedure can be repeated. The organic layers are then combined and by evaporation of the organic medium the analyte can be confined to a small volume of solution.

Mieure and Dietrich [61] used 15 mL of methylene chloride (dichloromethane) per 500 mL of aqueous solution. Their method involved first saturating the aqueous

solution with sodium chloride to improve the extractability of the water soluble compounds. After separation of the two phases about 10 mL of the organic phase was typically recovered which, potentially, gives a 50 fold increase in the concentration of the analytes. This solution was then analysed by GC-MS. Using this method they demonstrated the presence of a variety of pollutants, including phenols, nitro compounds, carboxylic acids and aromatic amines in wastewater, all in concentrations as low as 0.2 mgL^{-1}. Similarly Hites [62], using LLE with dichloromethane and subsequent GC-MS identification of the analytes, demonstrated the presence of phthalate esters in New England rivers and persuasively linked their presence to specific factories in the immediate neighbourhood. A continuous LLE method employing dichloromethane has been described by Kolpin *et al.* [23] for the extraction of a variety of wastewater contaminants including BPA, dieldrin, lindane, 4-methylphenol and 4-nonylphenol. Siegener [63] and Gardinali [24] used LLE with dichloromethane to estimate the concentration of caffeine in water, the former working on the seawater of Boston harbour.

1.2.2: Background to the technique of solid phase extraction

Solid phase extraction (SPE) is a technique that can trace its origin back to the 1950s. At that time the use of granulated active carbon (GAC) for the treatment of domestic water supplies was an established technique. Braus *et al.* [64] adapted this technique for analytical purposes. They built a system that could contain between 1200 and 1500 grams of GAC in iron cylinders that measured about 3 ft in length with an internal diameter of 4 inches. With this system they could sample large volumes of water (e.g. 80,000 gallons) at a flow rate of between 0.1 and 0.6 gallons per minute. Soxhlet extraction with diethyl ether was used to remove the adsorbed material from the carbon. After evaporation of the ether, sequential extraction of the remaining material with acidic and alkaline solutions of varying pH allowed its separation into phenolic, acidic, neutral, basic and amphoteric components.

Rosen *et al.* [65] used the method of Braus to sample river and canal water for petroleum components. They isolated the neutral material (i.e. they disregarded any phenolic, basic or acidic components) from that which had been adsorbed onto the active carbon and then fractionated this material on silica gel using a sequence of aliphatic (2,2,4-trimethylpentane), aromatic (benzene), and polar (chloroform/methanol) solvents. In this way they isolated aliphatic, aromatic and polar compounds from the residue. Using infra-red (IR) spectroscopy they compared these extracts with typical petroleum waste. By applying the method to river and lake waters from different locations they were able to estimate the contribution that petroleum waste was making to the total hydrocarbon content of those waters.

Rosen and Middleton [66] used GAC to extract chlorinated insecticides such as benzene hexachloride, aldrin, chlordane, methoxychlor, and DDT from large volumes (5000 gallons) of water. The insecticides were separated from the bulk adsorbed material by chromatography on active alumina using chloroform as the solvent. The insecticides were readily eluted whereas many oxygen containing organic compounds that caused IR interference were retained. Subsequently the insecticides were identified by comparison of the IR spectra of these extracts with the IR spectra of standard samples of the insecticides.

By the 1970s it was apparent that there were limitations associated with the use of charcoal as a sorbent. Amongst the difficulties that are frequently referred to are the occasional chemical modification of the analyte after elution and problems in eluting some analytes from this material. Grob [67] considered these problems but nevertheless selected charcoal to sample potable water, believing that an "..... advantage of charcoal, especially in comparison with synthetic adsorbents, is its absolute chemical stability. Under no conceivable conditions does it release substances that would result in contamination". He emphasized that the possibility of chemical modification of the analytes was exacerbated by the presence of certain inorganic salts in the charcoal that acted as centers for such modifications. Thus the selection of pure charcoal was of the utmost importance. That which he selected was a brand that had been developed for use in cigar filters. Using this

material potable water from the Zurich, Switzerland, supply was sampled and shown to contain a range of hydrocarbons, many of which were derived from automobile fuel.

1.2.3: Synthetic resins

One of the factors that contributed to the difficulties encountered with the use of GAC was the heterogeneous nature of this material. In the mid 1960s Rohm and Haas Co introduced a crosslinked polystyrene resin, Figure 1.1, Amberlite XAD-1. This material is far more homogeneous than GAC, so these problems were not encountered. Riley [68] used this material to preconcentrate organic compounds from water supplies. In the years to come various modifications of this resin would be introduced, Amberlite XAD-2, XAD-4, etc.

Recognising the limitations of charcoal as a sorbent of organic pollutants, Burnham *et al.* [69] examined the ability of the macro-reticular resin, Amberlite XAD-2 to adsorb a variety of pollutants from drinking water. Using a column that measured 1.5×7.0 cm and that was filled with this material they sampled 150 L of well water in Iowa, USA, at a flow-rate of 50 $mLmin^{-1}$. The retained material was eluted with diethyl ether. A variety of techniques that included UV-spectroscopy, IR-spectroscopy, and GC-MS were used to identify and to quantify components of this material. Amongst the compounds that were detected were acenaphthalene (19.3 μgL^{-1}), 1-methylnaphthalene (11.0 μgL^{-1}) and indene (18.0 μgL^{-1}).

In the late 1970s van Rossum and Webb [70] measured the recovery efficiencies of XAD resins (XAD-2, -4, -7 and -8) using distilled water samples spiked with thirteen organic pollutants that included 2-benzothiazole, p-cresol, dibenzofuran, 1-methylnaphthalene, phenol and α-terpineol. Identification and quantification of the analytes was achieved using GC with a flame ionization detector and an appropriate internal standard. The % recovery of the individual compounds was averaged and this mean value was used as a measure of the best resin. For

comparison, LLE with chloroform was also carried out. This latter procedure gave 80% recovery whereas the best individual resin was XAD-2 which gave 71%. A mixture of XAD-4 and XAD-8 gave a recovery of 76%. Using this mixture of resins they sampled 1000 L of Athens tapwater and extracted and quantified a range of both aliphatic and aromatic hydrocarbons that included n-dodecane (0.009 μgL^{-1}), n-tridecane (0.006 μgL^{-1}), naphthalene (0.009 μgL^{-1}) and anthracene (0.255 μgL^{-1}).

The synthetic resins proved to be effective sorbents and they quickly displaced the use of carbon as a material for measuring low levels of organic compounds in water. However when they first came on the market it was essential to clean them before use of residual material remaining from the manufacturing process. Junk *et al.* (71) systematically tested the efficiency of XAD-2 in recovering a wide range of compounds from water but their method involved cleaning the resin before use by sequential Soxhlet extraction of the resin with methanol, acetonitrile and diethyl ether continuing the extraction for eight hours for each solvent.

1.2.4: Bonded silicones

The work of Aue *et al.* [72] indicates that support bonded silicones $(C_{18}H_{37}SiO_{3/2})_n$ had also been prepared in the early 1970s and had been used to extract trace amounts of organochlorines from aqueous solution. Their work included the extraction of 10 L of water that had been spiked with a range of insecticides that included dieldrin, aldrin, heptachlor and lindane, all at concentrations in the low ngL^{-1}. Subsequently the retained analytes were eluted from the column with pentane and identified and quantified by comparison with standards using GC with electron capture detection. They applied their method to Missouri river water with inconclusive results.

During the 1970s the development of SPE technology was aided by techniques that were being developed by those working with HPLC, e.g. Little and Fallick [73] describe a method that they refer to as "trace enrichment". The challenge that they faced was to increase the concentration of an analyte to a point at which the

detectors then available could detect that analyte. They pumped the analyte in aqueous solution through the reversed phase column (e.g. C18). The analyte accumulated on the column and could subsequently be eluted by changing to a stronger solvent.

Modified silica with reversed-phase sorbent is one of the most widely used packing materials for SPE. Hydrophobic interactions between the C18 chains and the analyte molecules (i.e. van der Waals forces) effect the retention of these molecules. Such materials have a narrow pH stability range, so when SPE must be carried out in strongly acidic or basic conditions, reversed-phase polymeric sorbents (PS-DVB) are used. These materials are particularly suitable for aromatic analytes because of the possibility of π-π interactions.

1.2.5: Substituted polystyrene di-vinyl benzene (PS-DVB)

A problem with porous polystyrene when aqueous solutions are being used is that of poor contact between the solution and the hydrophobic surface. It is, therefore, necessary to wet the surface with a solvent such as methanol, acetonitrile or acetone. Though this procedure does give a better surface contact, the activating solvent can be gradually leached out of the sorbent causing extraction to be ineffective. A better approach is to bond hydrophilic moieties to the resin. A variety of substituted PS-DVB polymers have been suggested some of which are commercially available (Figure 1.2). A further advantage that is conferred by the introduction of substituents to the PS-DVB lattice is an increase in its capacity for more polar analytes.

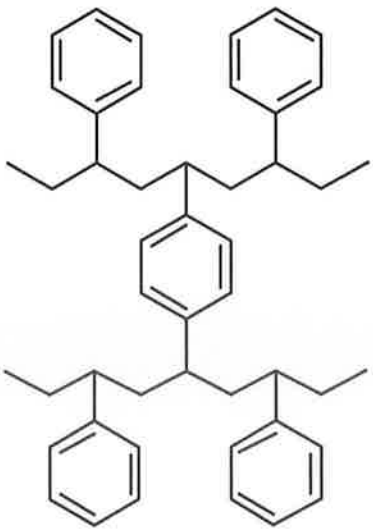
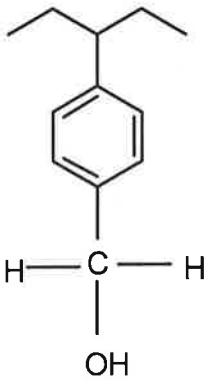
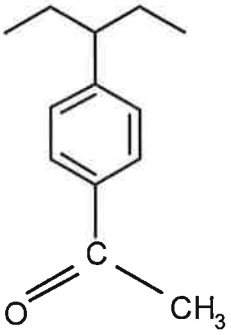


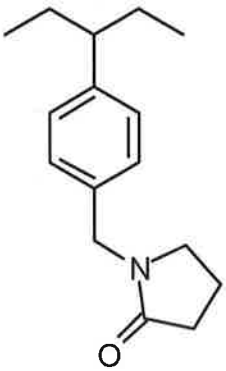
Figure 1.1: Structure of polystyrene di-vinyl benzene (PS-DVB), a cross linked polymer.



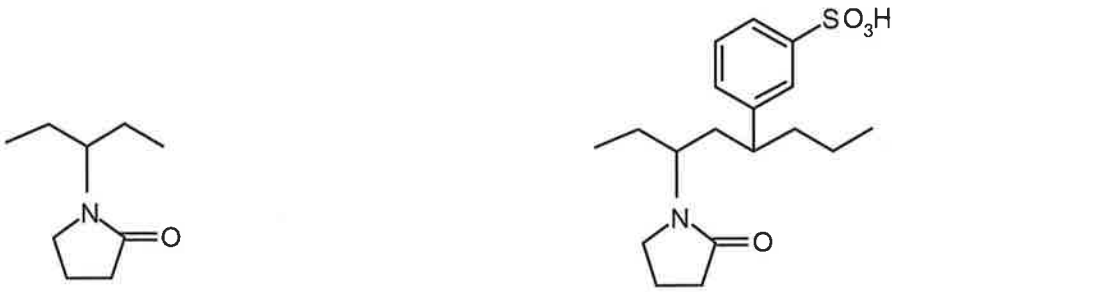
Acetyl



Hydroxymethyl



Pyrrolidone (as in Strata-X)



Pyrrolidone (as in Oasis HLB)

Pyrrolidone with sulphonic acid groups on the benzene rings (as in Oasis MCX)

Figure 1.2: Some examples of substituted PS-DVB

1.2.6: Retention mechanisms in solid phase extraction

The forces by which an analyte will be adsorbed by a solid phase extraction (SPE) sorbent are dependent upon the nature of the sorbent. Two types of sorbent will be considered, reversed phase (RP) type sorbents and ion exchange type sorbents. In the RP type of sorbent, e.g. C18, adsorption of very slightly polar molecules is effected by van der Waals forces (Figure 1.3). Elution can then be effected with a moderately polar solvent such as methanol or acetonitrile.

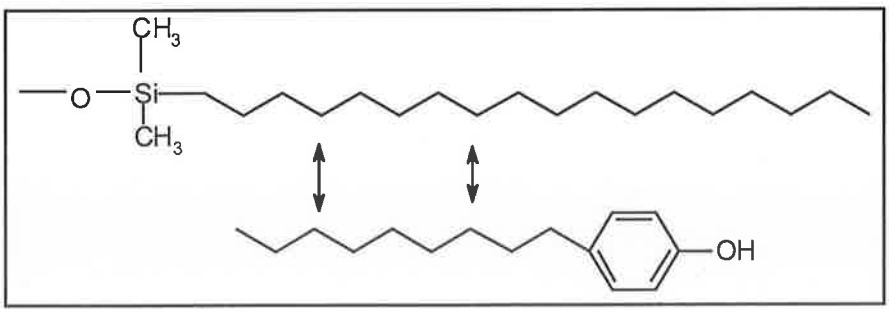


Figure 1.3: Retention of the analyte, 4-nonylphenol by the RP C18 sorbent. (Arrows represent van der Waals forces).

In ion exchange sorbents retention occurs through Coulombic attractive forces. There are, broadly, two types of such materials, cation exchanging sorbents and anion exchanging sorbents. As the names suggest these are used to extract cationic and anionic species respectively. For each of these types of sorbent careful consideration must be give to the respective pK_a values of the sorbent and of the analyte and the pH of the extraction medium must be controlled accordingly.

Recalling that $pH = pK_a \Rightarrow [BH^+] = [B]$, the following example, Figure 1.4, illustrates the situation for the Bronsted base, B and it conjugate acid BH^+.

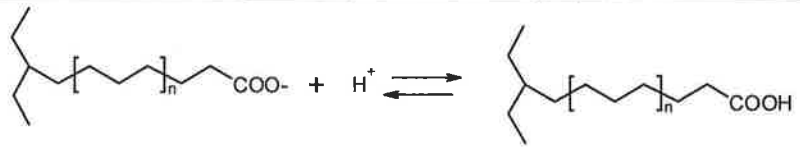
Sorbent (Cation exchanger)	Analyte
	$BH^+ \rightleftarrows B + H^+$

Figure 1.4: Respective equilibria of a carboxyl type sorbent and protonated base

Presuming that the pK_a value of the sorbent is 5 an effective working pH for this sorbent would be 7 (at this pH value the sorbent would be predominantly ionised and capable of effecting a coulombic attraction on any cations in solution). To achieve a predominantly ionised form of the type of analyte shown at this pH value the pK_a value of the analyte would need to be 9 or greater. Such protonated molecules are very weak acids, or, they are the conjugate (Bronsted) acids of very strong bases.

A corresponding situation can be described for anion exchanging sorbents, Figure 1.5.

Again, $pH = pK_a \Rightarrow [HA] = [A^-]$

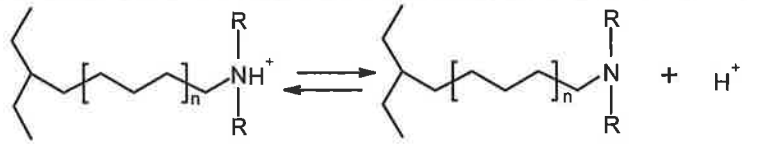
Sorbent (Anion exchanger)	Analyte
	$HA \rightleftarrows H^+ + A^-$

Figure 1.5: Respective equilibria of an ammonium type sorbent and a weak acid

If the sorbent has a pK_a value of 9 its working pH would be 7. Thus the required pK_a values of analytes of the form shown would be 5 or less.

In general, elution of analytes from ion exchange sorbents can be effected by reversing the equilibria which ensured the retention of those analytes.

It is important to note that many commercially available sorbents combine both a RP and an ion exchange facility within their structure, e.g. Oasis MCX, above, Figure 1.2.

1.2.7: The technique of solid phase extraction

SPE sorbents can be employed in different ways. These include cartridges, discs [74] and stir-bar methods [75]. The use of cartridges will be described. An SPE cartridge is the barrel of a hypodermic syringe in which a definite mass of the sorbent is contained at the bottom of the cartridge between two teflon or polyethylene frits. The sample is contained in the barrel above the sorbent and is brought through it by means of a pressure differential. As the sample passes through the sorbent the target analyte(s) are selectively adsorbed. Subsequently they can be eluted from the sorbent by means of an appropriately chosen solvent or, in the case of ion exchange sorbents, by an appropriate adjustment of the pH value. An SPE protocol, typically, involves five steps. These are as follows: conditioning of the sorbent, equilibration, loading of the sample on the sorbent, washing of the sorbent to remove unwanted coextractives and elution of the analyte from the sorbent.

Conditioning of the sorbent: this is achieved by running a small volume of an organic liquid, e.g. methanol, through the sorbent. Its effect is to "wet" the hydrophobic sorbent.

Equilibration of the sorbent: a small volume of water is then passed through the sorbent.

Loading of the sorbent with the sample: an aqueous solution of the analyte(s) is then passed through the sorbent. Depending upon the respective nature of the sorbent and of the species in solution some molecules will be retained and others will pass through, e.g. hydrophobic, non-polar molecules would be retained by a C18 sorbent, but more polar, lipophobic species would pass through.

Washing of the sorbent: certain unwanted species can be removed from the sorbent by careful selection of a suitable wash, e.g. aqueous methanol will remove substances from a C18 sorbent that were sufficiently lipophilic to be retained during the loading process, yet of greater polarity than the desired analyte which will remain adsorbed.

Drying of the sorbent: before elution of the analytes the sorbent is thoroughly dried by means of a flow of air or of nitrogen. This is considered necessary as the presence of water could hydrolyse certain analytes or impede their elution by hindering access of hydrophobic eluants to the sorbent.

Elution of the analyte: The analytes are removed from the sorbent with a suitable solvent, e.g. methanol or acetonitrile can be used to elute non-polar molecules from a C18 sorbent.

For quantitive purposes this eluate can be reduced to dryness by evaporation and then made up to a precise volume prior to analysis.

The steps loading, washing and elution are illustrated in Figure 1.6.

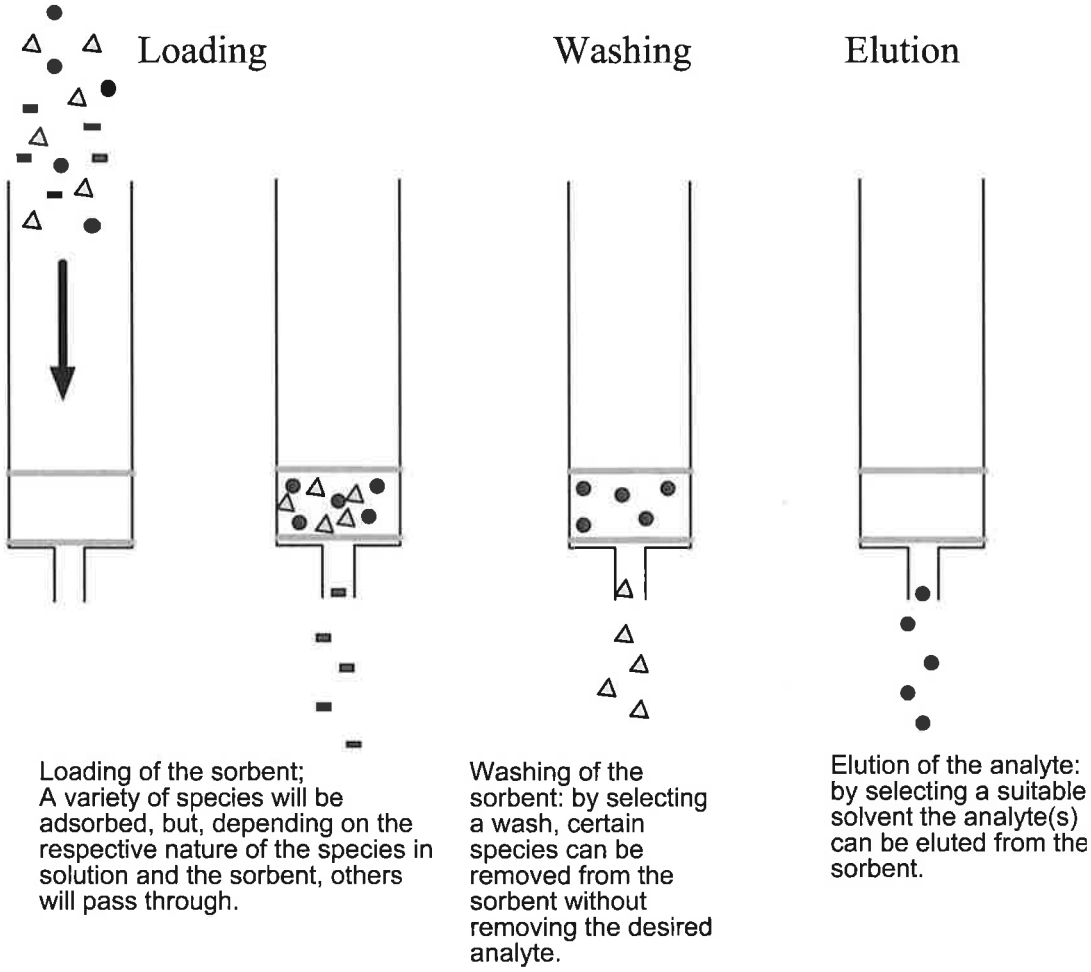


Figure 1.6: Diagrammatic representation of the process of solid phase extraction

1.2.8: Reported SPE methods

The SPE technique that has been described will, of course, be modified to take account of the volume of the sample, the target analytes and the chosen sorbent. Some reported protocols are described below.

Verenitch *et al.* [76] targeted caffeine and acidic drugs, including ibuprofen, in surface water and municipal waste water. They collected 2 L samples and split these into 1L aliquots one of which was used for the analysis of acidic drugs and the other for the analysis of caffeine. The sample which was to be analysed for acidic drugs was adjusted to pH 2.0 with sulphuric acid to ensure their presence in the non-ionised form and then extracted with 500 mg of the sorbent SupelcoLC-18 (an octadecyl bonded, end capped silica) that had been conditioned with 6 mL hexane, 3 mL acetone, 6 mL

dichloromethane (DCM), 2 mL water and 2 mL water (pH 2). The flow rate through the sorbent was at 10 $mLmin^{-1}$. After loading, the sorbent was dried for 2 min and then eluted with 3×3 mL of methanol. This extract was then reduced to 0.2 mL with nitrogen gas prior to derivatisation and analysis by GC-MS. The 1L aliquot to be analysed for caffeine was adjusted to pH 7.5 with aqueous sodium hydroxide prior to extraction using Oasis HLB cartridges (200 mg). These were conditioned with 3 mL methyl *tert*-butyl ether (MTBE), 3 mL methanol and 3 mL water. The flow rate through the sorbent was at 10 $mLmin^{-1}$.

After loading the sorbent was washed with 2 mL 25% aqueous methanol to remove any polar coextractives. The adsorbed analytes were eluted with 1 mL of methanol followed by 6mL methanol/MTBE(1:9 v/v) and then reconstituted with 0.1 mL light petroleum prior to analysis by GC-MS. A method detection limit (MDL) of 0.8 ngL^{-1} for ibuprofen and 20 ngL^{-1} for caffeine is reported.

Farre *et al.* [77] used a commercially available bioassay product and chemical analysis to identify toxic compounds in waste water. The latter involved the same sorbent (LiChrolut RP-18, 500 mg) but different protocols to target a variety of chemicals that included estradiol, ethynylestradiol and nonylphenol. The procedure to isolate the estrogens involved conditioning the cartridges with 7 mL of acetonitrile and 5 mL of water and using them to extract 500 mL of the sample at a flow rate of 5 $mLmin^{-1}$. After loading the sorbent was dried for 20 – 30 minutes and then eluted with 2×4 mL of acetonitrile. The extracts were then blown down to 0.5 mL with nitrogen gas and analysed by LC-DAD-MS.

The phenols were targeted by conditioning the sorbent with 7 mL methanol and 3 mL of water and then extracting 200 mL of sample at a rate of 5 $mLmin^{-1}$. The cartridges were then dried and the adsorbed analytes were eluted sequentially with solvent mixtures of increasing polarity. Using this method the authors failed to detect estrogens in the wastewater treatment plant (WWTP) influent or effluent but nonylphenol was detected in the final effluent of two WWTPs at concentrations of 5.5 and 6.6 μgL^{-1}.

Quintana *et al.* [78] report a procedure for the estimation of natural and synthetic estrogens in waste water. They conditioned Oasis HLB cartridges with ethyl acetate, methanol and water and used them to extract 1000 mL of waste water at a flow rate of 15-20 $mLmin^{-1}$. After extraction the cartridges were dried for 30 min and then eluted with 3 mL ethyl acetate. Analytes in the resulting extracts were derivatised and then quantified by GC-MS. A method detection limit (MDL) of 2 ngL^{-1} for estradiol and 3 ngL^{-1} for ethynylestradiol is reported. When the method was applied to the influent of a sewage water treatment plant (SWTP) ethynylestradiol was not detected but estradiol was detected at a concentration of 13.1 ngL^{-1}.

In this report [78] the authors recall the observation of previous workers that, though the extraction of estrogens is favoured by a low pH such conditions facilitate the extraction of humic acids from environmental samples by reversed phase (RP) sorbents yielding extensively contaminated samples. The influence of pH on the recovery of these compounds was investigated. It was found that the recovery rate was similar at pH 2, 6 and 8. Hence they recommended extraction without pH adjustment.

They also investigated the breakthrough of the analytes by coupling two cartridges in series. After extracting different sample volumes they investigated the analytes retained by each. Analytes were not detected in the second cartridge even after the concentration of 2 L of Milli-Q water, river water or sewage effluent spiked with the hormones at a concentration of 5 ngL^{-1} though in the latter case blockage of the cartridges occurred after about 1500 mL of sample had passed through.

De Alda and Barcelo [79] have reported the % recovery of several steroid sex hormones using three different reversed phase (RP) sorbents. In each case the cartridges were conditioned with 7 mL acetonitrile, 5 mL of methanol and 5 mL of water. Filtration through glass fibre filters (0.45 μm) of the waste water samples prior to extraction was considered essential. Considering the length of time needed for the filtration 500 mL sample volumes were found to be the most satisfactory. After loading, the sorbents were dried for 20-30 minutes and then eluted with 2×5 mL

acetonitrile. This was found to be a more effective eluant than DCM or methanol. The extracts were blown down to dryness with nitrogen gas and then reconstituted to 0.5 mL with methanol.

In the extraction of several analytes that included estradiol and ethynylestradiol from distilled water spiked with each analyte at 10 μgL^{-1} the three sorbents, Isolute ENV, LiChrolut EN and LiChrolut RP-18 exhibited similar retention behaviour towards all analytes but estriol.

Ahrer, Scherwenk and Buchberger [80] have reported a method for the estimation of drug residues, including ibuprofen, in water. The optimised SPE procedure involved conditioning 500 mg Bondesil ODS in 6 mL cartridges with acetone, methanol and water at pH 2 and using them to extract 500 mL of water that had been adjusted to pH 2 with sulphuric acid. The flow of the sample through the cartridge was at about 10 $mLmin^{-1}$. After loading, the cartridges were dried for 30 min and then the adsorbed analytes were eluted with 2 mL of methanol. These extracts were reduced to dryness with nitrogen gas and then reconstituted with methanol and analysed with LC-MS or CE-MS. Based on the average recovery of nine drugs the sorbent Bondesil ODS was found to be superior to four other sorbents that included Oasis HLB. The influence of sample pH was checked and it was found that pH 2 gave a better recovery than pH 5.5 or pH 8.5.

The importance of silanising all glassware was emphasised as this prevents adsorption of analytes which would otherwise considerably reduce the % recovery. A recovery rate of 90.2% for ibuprofen is reported and a MDL of 0.6 ngL^{-1} when analysed by HPLC-MS.

Kosjek *et al.* [81] have reported an analytical procedure that can be used to estimate common non-steroidal anti-inflammatory drugs (NSAID), including ibuprofen, in waste waters. Their method involved filtering 1 L samples with 0.45 μm filters, acidification to pH 2.6 and extraction with Strata-X (60 mg, 3 mL) cartridges that had been conditioned with 1.5 mL methanol and 1.5 mL water acidified to pH 2.6. After extraction the cartridges were dried for 1 min and the adsorbed analytes were eluted

with 3×0.5 mL methanol. The resulting extract was brought to dryness with nitrogen gas and then reconstituted. The contained analytes were derivatised and analysed by GC-MS. Using this method the % extraction of ibuprofen was reported at 84.2 and 91.9 depending on the initial concentration of the loading solution and the LOQ of the method (for ibuprofen) was 1.96 ngL^{-1}.

Castiglioni *et al.* [82] report a method developed for the simultaneous estimation of 30 pharmaceuticals belonging to various therapeutic categories in urban waste water. Amongst the pharmaceuticals considered were estradiol, ethynylestradiol and ibuprofen. The method utilised the SPE sorbent Oasis MCX (poly-divinylbenzene-co-N-vinylpyrrolidone with sulphonic acid groups on the surface) that had been conditioned with 6 mL methanol, 3 mL water and 3 mL water at pH 2.

Samples (500 mL) were extracted at a flow rate of 20 $mLmin^{-1}$. After loading, the sorbent was dried for 5 min and then eluted with 2 mL methanol, 2 mL 2% ammonia solution in methanol and 2 mL 0.2% sodium hydroxide in methanol. Subsequently the extracts were analysed by HPLC-MS. Using this method recovery rates of 92% (estradiol), 81% (ethynylestradiol) and 91% (ibuprofen) and LOQ of the method of 5.2 ngL^{-1} (estradiol), 4.6 ngL^{-1} (ethynylestradiol) and 1.38 ngL^{-1} (ibuprofen) were reported.

When the method was applied to the effluent of several Italian STPs a number of pharmaceuticals were detected, some in concentrations of over 2000 ngL^{-1}. However, neither estradiol, ethynylestradiol nor ibuprofen were detected.

1.3 Sampling procedures

The distribution of anthropogenic pollutants within a waterway, (river, lake or sea) is a phenomenon that may be consistent throughout the waterway or it may manifest definite local variations.

In the former case the pollution might be the persistent legacy of a bygone incident, e.g. the presence of difocol in Lake Apokpo, Florida. The latter situation will usually arise when there is a source, e.g. outfall from a WWTP or a factory discharge, that is constantly adding pollutants to the waterway. In this case the concentration of the pollutants will be high in this immediate neighbourhood but will diminish appreciably, perhaps approaching a background level, as distance from the source increases.

A concentration gradient will arise spontaneously from the process of diffusion, but this will be markedly influenced by such factors as river flow, tidal effects, surface evaporation and any barriers, either natural or artificial that may be present.

In some cases adsorption of the pollutant to sedimentary particles may be extensive and an equilibrium may exist between such molecules and those within the aqueous phase.

Sampling schemes for such waterways will seek to achieve a set of representative results from which useful statistics may be inferred. Such schemes may involve the random selection of sampling sites or a more deliberate targeting of certain sites based upon extensive and intimate knowledge of the area, e.g. Roberts and Thomas [83], investigating the occurrence of pharmaceutical residues in wastewater effluent and surface waters of the lower Tyne catchment targeted six sites along the estuary, choosing sites that would be accessible at low and at high tides. They collected samples at each site at intervals of 2 hours over a 12 hour period and combined these to give a composite sample from each site. Beck *et al.* [40] in a study of estrogenic pollution of the Baltic Sea based their selection of sampling sites on the known presence of fauna that exhibited morphological anomalies presumed to have been caused by such pollution. Such a targeted approach can be designed to be

representative of the waterway, however, it is always open to the possibility of an unconscious bias in the site selection.

A random selection of sites is one in which each potential site has an equal chance of being selected. This approach has the advantage of avoiding any systematic bias that might otherwise be present in the selection, however it does not, necessarily, yield a representative selection scheme.

Random sampling of such a waterway would be achieved by imposing a numbered grid on a map of the waterway, writing each number on identical slips of paper and drawing a definite number of these slips from a hat. Alternatively, a table of random numbers may be used. A rule of thumb that has been suggested for the number of sites that should be selected is $(\sqrt{N}) + 1$, where N = the total number of potential sites.

1.4: Aim and objectives of the project

The project aimed to identify one pollutant of environmental waters the concentration of which might be used as an index for the concentration of other anthropogenic pollutants. To achieve this aim it was necessary to approach the following objectives:

1. To select a body of water, river, lake or estuary, in a populated area that receives the wastewater from this population, either treated or untreated.
2. To select a set of sampling sites that would be representative of the selected water body.
3. To select a number of anthropogenic molecular markers as target compounds.
4. To develop methods of extracting, identifying and quantifying these marker compounds in a matrix that would be comparable to that of the chosen water body.
5. To apply these methods to the extraction, identification and quantification of these compounds in water taken from each of the selected sampling sites.
6. To identify any relationship that might exist between the concentrations of these anthropogenic pollutants in such a way that one pollutant might be identified as a possible index of others.

Chapter 2

Geographical background to the project, development of a sampling plan and selection of target compounds

2.1: Geographical background to the project

2.1.1: Location of the project

The Dublin region, comprising Dublin City and County, is an important location for manufacturing in the Republic of Ireland, with 1273 manufacturing companies registered in the year 2000, accounting for 28% of the countries industrial output. Leading manufacturing sectors are metal products, electronics and engineering, chemical, rubber and plastics products and paper and publishing. The high growth sectors have been in paper, paper and publishing, electronics, pharmaceuticals and chemicals with the textile industry in decline. In the year 2002 the total number of people at work in manufacturing industry in the Dublin region was 54412. In recent years the pharmaceutical/chemical industry has become increasingly important in the Dublin region. Dominated by multinational companies it is mainly concerned with the manufacture and the distribution of research based pharmaceuticals, adhesives, fine chemicals and bulk chemicals. Major companies operating in the Dublin region include Glaxo-Smith Kline, Yamanouchi (Mulhuddart), Organon (Swords), Helsinn Chemicals (Mulhuddart), and Bristol Myers Squibb (Swords). Plastics are not made in Ireland, however there is blow moulding, extrusion and thermoforming of between 220,000 and 250,000 tonnes/annum.

Six million visitors come to Ireland each year [84], a large proportion of whom come to the Dublin region. In the period 1996-2002 the population of Dublin increased by more than 5% and that of Meath and Kildare by over 20% and the population of the Republic of Ireland reached 4,000,000.

Dublin Bay comprises an area of over 80 square kilometers. It lies between the Latitudes 53° 16' and 53° 22' and the Longitudes 06° 13' and 06° 02'. It is bounded to the north by the suburbs of Clontarf, Sutton and Howth and to the south by Blackrock, Dun Laoghaire and Dalkey. Several waterways flow into the bay including the River Liffey, the Grand and the Royal Canals, the River Tolka and the River Dodder.

There is a major wastewater treatment plant (WWTP) at Ringsend that accepts sewage and waste water from North Dublin and from South Dublin. Traditionally sludge from this plant was dumped at sea near Howth Head however since 2003 this sludge has been treated by sterilisation at 450°C and is now marketed as fertiliser. Treated water from the Ringsend plant is returned to the sea just north of the South Bull Wall. This wall is a rampart built in the 18^{th} century to prevent the accumulation of silt in the mouth of the river Liffey and thus ensure access to Dublin port. It extends seawards a distance of 3 km.

2.1.2: Selection of potential sampling sites

Figure 2.1 is an extract of the Ordnance Survey map of the Dublin region (Scale 1:20000, 50mm = 1km). Along the coastline from the Martello Tower just beyond Sutton to Sorrento Point, Dalkey the existing 1km squares on this map were subdivided into four squares of area 0.25 km^2 as shown in this figure. This yielded over 112 potential sampling sites. Some of these sites were necessarily excluded from consideration since access to the sea or river was either dangerous or in private ownership.

2.1.3: Random selection of 15 sampling sites

One hundred and twelve suitable sampling sites were identified and numbered. A random selection of 15 of these sites was made as follows: the columns and the rows in a table of random numbers were numbered. Two random numbers were generated by calculator to give a starting point on the table. The 15 numbers ≤ 112 were then selected by proceeding sequentially through the column of numbers.
The sites that were selected in this way are listed in Table 2.1 with grid references and are shown in Figure 2.1.

2.1.4: Photographs of the sampling sites

Photographs of the sampling sites are shown in Plates 2.1 to 2.14

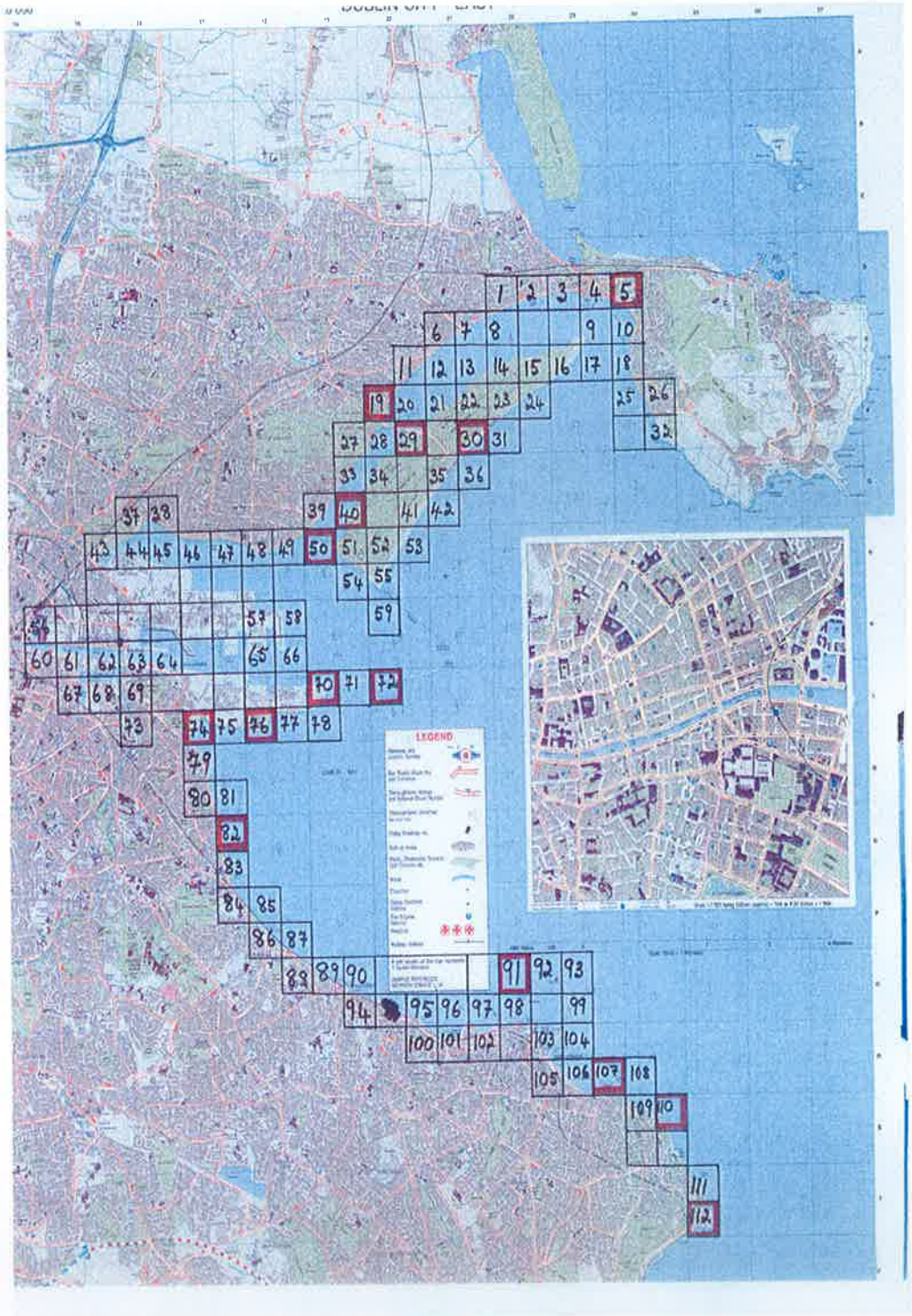


Figure 2.1: Map of Dublin Bay showing 112 potential sampling sites of which 15 were randomly chosen for the collection of triplicate samples. Each sampling site measures 500 × 500 m^2. The randomly selected sites are outlined in red. (A larger scale map is shown inside back cover)

2.1.5: Sampling model

The mean values of statistics of samples taken at random from these sampling sites were used to estimate those statistics of the water at each site. Three 2.7 L samples were taken at random from each sampling site.

Table 2.1: List of sampling sites, site numbers as shown on map, Figure 2.1, and grid references as defined by Ordinance survey map (1:50,000), Discovery Series 50

Sampling Site	Site Number	Grid Reference
Sutton strand	5	O 262 391
Causeway (north)	19	O 223 375
Causeway (south)	29	O 226 373
Dollymount strand	30	O 236 369
Behind house on Bull Island	40	O 218 365
Bridge to Bull Island	50	O 214 358
Start of South Bull Wall	70	O 215 338
Half Moon Swimming Club (north)	72	O 225 338
Strand near Sean Moore Park	74	O 193 333
Strand near Irishtown Nature Park	76	O 207 334
Merrion Strand	82	O 196 317
West Pier (Dun Laoighaire)	91	O 244 297
Sandycove Harbour	107	O 258 282
Bullock Harbour	110	O 263 278
Sorrento Point (park at junction of Nerano Road and Coliemore road)	112	O 273 263

Plate 2.1: Site 5, Sutton strand

Plate 2.2: Site 19, northern side of the causeway to the Bull Island, Dollymount

Plate 2.3: Site 29, Southern side of the causeway to the Bull Island, Dollymount

Plate 2.4: Site 30, Beach at Dollymount

Plate 2.5: Site 40, Northern side of the bridge across to The Bull Island

Plate 2.6: Site 50, Southern side of the bridge across to the Bull Island

Plate 2.7: Site 70, the South Wall of Dublin Port. Site 72 was at the Half Moon Bathing Club premises which can be seen in the distance along the wall

Plate 2.8: Site 76, Beach just south of the Poolbeg ESB power station, bordered by the Nature Reserve

Plate 2.9: Site 82, Sandymount Strand looking across towards the ESB power Station

Plate 2.10: Site 91, West Pier, Dun Laoighaire Harbour

Plate 2.11: Site 107, Sandycove Harbour

Plate 2.12: Site 110, Bulloch Harbour, Dalkey

Plate 2.13: Site 112, Dalkey Island, seen from the park at Sorrento Point

Plate 2.14: Point of discharge of the WWTP effluent into the cooling water flowing from ESB power station, and thence into the water of Dublin Bay

2.2 Target compounds

2.2.1: Selected target compounds

The marker compounds chosen were, caffeine, estradiol, ethynylestradiol, ibuprofen methylparaben and 4-nonylphenol.

2.2.2: Information on target compounds

2.2.2.1: Caffeine

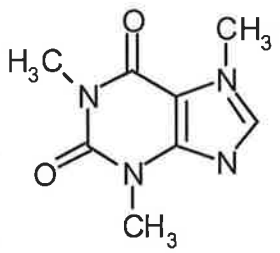


Figure 2.2: Structure of caffeine (1,3,7-trimethylxanthine)

The structure of caffeine is shown in Figure 2.2. This compound is a plant alkaloid found in the beans and in the leaves of certain plants. In these plants it acts as a natural pesticide, paralysing and killing any insects that feed upon it. In humans it acts as a central nervous system stimulant and is believed to ward off sleepiness. Its water solubility is 10-50 $mgmL^{-1}$ at 23 degrees Celsius. Its lethal dose for rodents (LD_{50}) is 127 $mgkg^{-1}$. This compares with a LD_{50} for DDT of 135 $mgkg^{-1}$ [60]. It occurs naturally in tea and in coffee and is found in some carbonated drinks, e.g. Coca Cola, Red Bull. It is available in the form of pills and is incorporated into some pharmaceutical preparations, e.g. Solpadeine. Caffeine is considered to be the world's most popular psychoactive substance. In the human body caffeine is metabolised into theobromine (80%), paraxanthine (11%) and theophylline (4%) [86], suggesting that about 5% may be excreted as unchanged caffeine. Each of these compounds is further metabolised, the eventual products being various substituted forms of uric acid. There are frequent reports of caffeine being found in environmental waters, see Table 2.2.

Table 2.2: Reported values for the occurrence of Caffeine in environmental matrices

Matrix (Country)	Concentration	Reference
River water (Holland)	500 ngL^{-1}	13
Sewage Influent (Norway)	104 μgL^{-1}	18
Sewage effluent (Norway)	0.07 μgL^{-1}	18
Seawater (North sea)	(2.0-16.1) ngL^{-1}	19
Surface Water (USA)	(0.003-1.44) μgL^{-1}	85
Estuary (Miami)	41.2 ngL^{-1}	24
Seawater (Biscayne Bay)	11.9 ngL^{-1}	24
Boston Harbour	(140 -1600) ngL^{-1}	63

2.2.2.2: Methylparaben

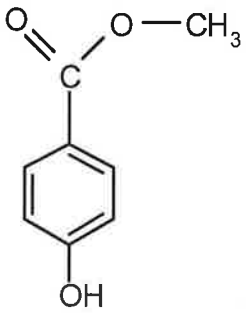


Figure 2.3: Structure of methylparaben

Parabens are the esters of *p*-hydroxybenzoic acid. They constitute an homologous series. Figure 2.3 shows the structure of the lowest member of the series, methylparaben. Other members are methyl, ethyl, propyl etc. *p*-hydroxybenzoic acid. They are effective antimicrobial agents, being particularly effective against moulds and yeasts. They are used in the preservation of certain foods, cosmetics and pharmaceutical preparations. The antimicrobial activity increases as the chain length of the ester group increases, however, solubility decreases with increasing chain length so the lower esters tend to be the ones most frequently used.

Methylparaben has a water solubility 0.25 g/100 mL. It is readily and completely absorbed through the skin and the gastrointestinal tract. Within the body it is hydrolysed to *p*-hydroxybenzoic acid, conjugated and the conjugates are rapidly excreted in the urine. In studies with rats, rabbits, dogs and cats the major metabolites detected in the urine were *p*-hydroxybenzoic acid, and the glycine, glucoronic acid and sulphuric acid conjugates of *p*-hydroxybenzoic acid. The parent compound was not detected in the urine [87]. Methylparaben has been used as a preservative in food and cosmetics for over 50 years and in pharmaceutical formulations for even longer. A variety of tests over many years have indicated that methylparaben is non-toxic either by ingestion or through skin contact [87].

The types of foodstuffs that may contain methylparaben include frozen dairy products, jams, jellies, pickles, processed fruits and vegetables, tomato pulp and fruit juices.

It has been claimed that methylparaben is the most frequently used preservative in cosmetics [87]. It is found in a wide variety of products including face and hand cream, lotions and moisturizers, eye makeup products, hair conditioners and shampoos and in deodorants.

Parabens were first employed as preservatives in pharmaceutical preparations in the 1920s [87]. They are used in injections (0.065-0.25%), ophthalmic preparations (0.015-0.05%), oral solutions and suspensions (0.015-0.2%), topical preparations (0.02-0.3%) and vaginal preparations (0.1-0.18%).

Despite the widespread use of methylparaben, to the knowledge of the author, no research seems to have been carried out into its occurrence in environmental waters.

2.2.2.3: Ibuprofen

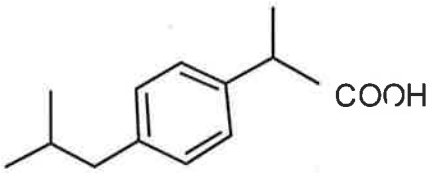


Figure 2.4: Structure of ibuprofen

Ibuprofen, (Figure 2.4), is a non-steroidal antiinflammatory drug that was developed by Boots in the early 1960s. It is marketed under several tradenames including, Nurofen and Brufen. It is used to treat minor aches and pains such as toothache, backache and the fever and pain that are associated with influenza. It can also be used to alleviate the pain associated with rheumatoid arthritis and primary dysmenorrhoea. Its use is characterised by a low incidence of gastrointestinal adverse drug reactions. It is available on prescription and over the counter (OTC). All of these considerations make ibuprofen a most widely used drug. There are frequent references to its occurrence in environmental waters (Table 2.3), including rivers [30, 23, 16 ,14], estuaries [20, 83], sewage [18,16,14] and seawater [18]. In a study of 84 streams across the US, Kolpin *et al.* [23] reported finding ibuprofen in 9.4% of the samples with a maximum concentration of 1.0 μgL^{-1}. In some regions widespread prevalence of the drug is indicated by appreciable concentrations being found upstream of a STW.

Table 2.3: Reported values for the occurrence of Ibuprofen in environmental matrices (ngL^{-1})

Matrix/Country	Concentration	Reference and Year of publication.
STW Effluent	(1700 -3800)	14 (2003)
Upstream	< 20	
Downstream	< 20	
(UK)		
STW Effluent	4201	16 (2004)
Upstream	432	
Downstream	1105	
(UK)		
Hospital sewer	380	18 (2004)
Seawater (Harbour)	0.3	
(Norway)		
Belfast Lough	124	20 (2004)
Thames Estuary	928	
Mersey Estuary	342	
(UK)		
Streams	1000*	23 (2002)
(USA)		
River Elbe	(5.1 -32)	30 (2004)
(Hamburg, Germany)		
Raw sewage	(7741 – 33,764)	83 (2005)
Tyne Estuary	(144 – 2324)	
(Howden, UK)		

* This is a maximum value in a National Reconnaissance.

2.2.2.4: 4-Nonylphenol

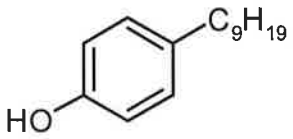


Figure 2.5: Structure of 4-nonylphenol. Technical nonylphenol includes molecules with different isomeric forms of the nonyl group

Figure 2.5 shows a structural type formula for the compound 4-nonylphenol. This is a refractory degradation product of non-ionic surfactants that have been synthesized by the action of ethylene oxide on the phenol. These surfactants are referred to as nonylphenol polyethoxylates and have the general formula shown in Figure 2.6.

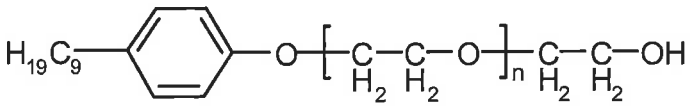


Figure 2.6: Structure of nonylphenol polyethoxylates. For industrial surfactants n is usually 9 or 10

The nonyl group may be any one of several different isomers. Technical 4-nonylphenol is a mixture of several isomers. Nonylphenol polyethoxylate surfactants have been in use in the UK since about 1944. In more recent years concern has been expressed regarding the affect that the degradation products of these materials might have on wildlife because of their endocrine disrupting potential. Though their use in domestic cleaning materials continues in certain countries, e.g. Taiwan [88], legislation prohibits their use for such purposes in Europe. Currently neither Proctor and Gamble, Unilever nor Johnson and Johnson use these materials in their products, though Proctor and Gamble have indicated that there are some cosmetic products from which the complete elimination of nonylphenol is proving difficult (http://www.pgperspectives.com).

In STWs alkyl phenols are degraded by the action of micro-organisms, as shown in Figure 2.7, successively losing ethoxy groups. In contrast with the parent surfactant, the free phenol is classified as an endocrine disruptor.

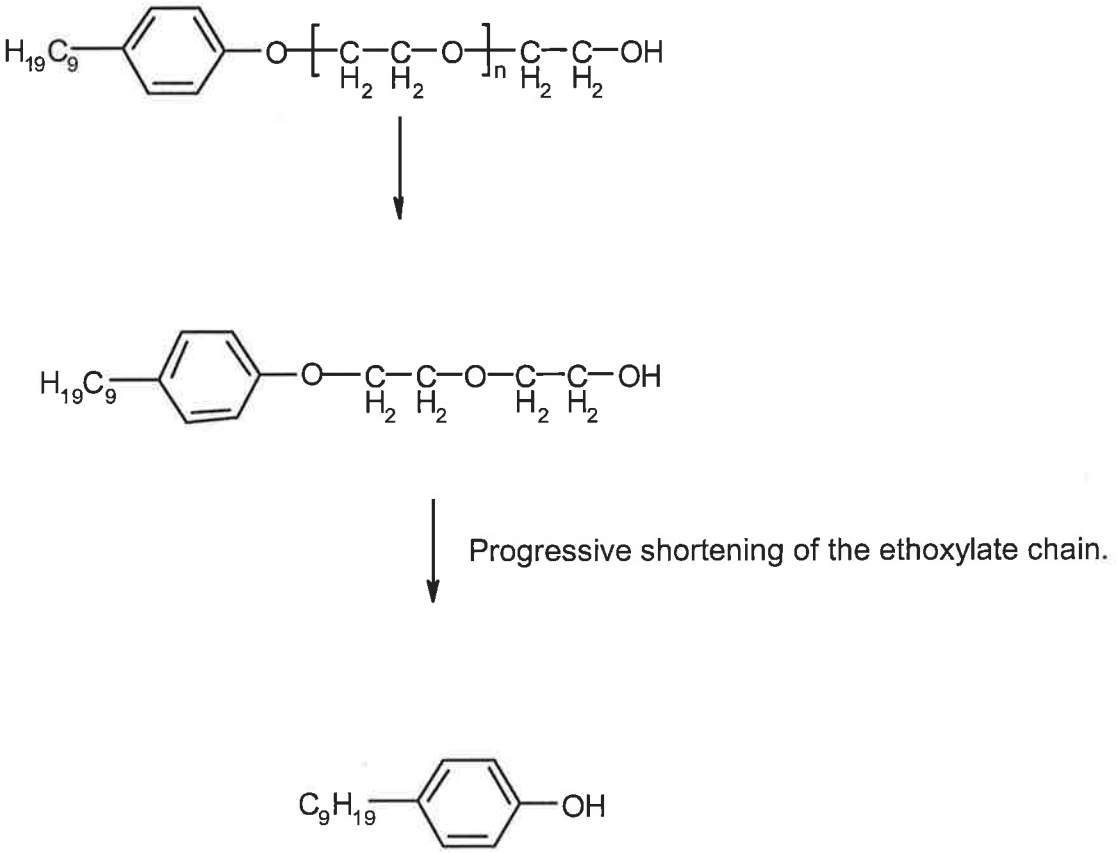


Figure 2.7: Diagrammatic representation of the degradation of polyethoxylate surfactant molecules effected by microbial activity in STWs

Though it has been shown that 4-nonylphenol shows preferential partitioning to the suspended solid phase [44] it has been found in environmental waters including estuaries and seawater. Table 2.4 summarizes some results that have been reported in the literature.

Table 2.4: Reported values for the occurrence of Nonylphenol in environmental matrices

Matrix (Country)	Concentration	Reference
Surface Water (Holland)	(<110 – 4100) ngL^{-1}	41
River Water (UK)	(3 – 215) μgL^{-1}	89
Estuaries (UK)	(< 0.2 -2.6) μgL^{-1}	90
Sewage (UK)	(< 0.8 – 4)ngL^{-1}	91
Streams (USA)	40 μgL^{-1}*	23

*This is a maximum value in a National reconnaissance.

2.2.2.5: Ethynylestradiol

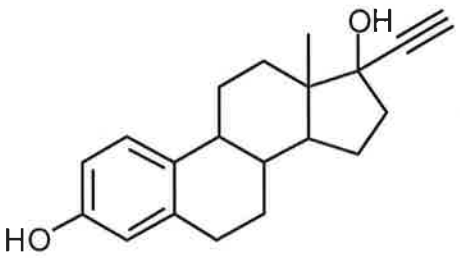


Figure 2.8: Structure of the semi-synthetic estrogen, ethynylestradiol.

The semi-synthetic estrogen, ethynylestradiol (Figure 2.8), was first synthesized in 1938 by Inhoffen and Hohlweg [92]. The properties of this compound are similar to those of the natural estrogen, estradiol, however, unlike this compound, it is not degraded by the digestive enzymes of the alimentary tract. Traditionally conditions arising from an estrogen deficiency, such as, amenhorrea or the symptoms of menopause, had been treated with estradiol administered orally or by intra-muscular injection. However, because of its susceptibility to digestion, the recommended oral dose was necessarily as much as ten times that of the intra-muscular injection [32]. The availability of ethynylestradiol offered to medical practitioners the opportunity of treating such conditions with a low dose of an oral formulation.

During the 1920s the Austrian physiologist, Haberlant, had shown that infertility in rabbits could be achieved by suppressing ovulation using ovarian extracts. When estrone and progesterone were isolated in the late 1920s and it was shown that these compounds could have the effect of suppressing ovulation in animal experiments efforts were made to exploit Haberlant's ideas for the purpose of developing an oral contraceptive formulation. However, since these compounds are readily digested like estradiol, ethynylestradiol was eventually selected as a compound suitable for this purpose.

Initially ethynylestradiol was prepared from estrone that had been isolated from the urine of pregnant mares. Since the anticipated demand for the compound was greater than could be met from this source an alternative raw material was needed. Russell E. Marker is credited with the discovery of the compound diosgenin in the roots of varieties of *Dioscorea* that grow wild in such countries as Mexico and Guatemala.

Using this compound as the raw material chemists were able to introduce the required molecular modifications and produce sufficient quantities of the synthetic estrogen, ethynylestradiol. Most oral contraceptive formulations now include this compound. Since the early 1980s those involved in such research have presented evidence of the occurrence of ethynylestradiol in environmental waters. For this compound the octanol/water coefficient is such that sorption to sludge is expected to remove much of the compound from the aqueous phase ($\log_{10}K_{ow} \sim 4.0$). Table 2.5 summarizes some of the results of such research.

Table 2.5: Reported values for the occurrence of ethynylestradiol in environmental matrices

Matrix/Country	Concentration (ngL^{-1})	Reference and year of publication
River Water (UK)	< 200	29 (1985)
River water (UK)	< 0.8	91 (2004)
STP effluent (the Netherlands)	(< 0.3 – 2.6)	41 (2004)
Rain water	< 0.3	
Surface water	(< 0.3 – 0.4)	
Seawater (Baltic Sea)	(< 0.005 – 17.2)	40 (2005)
River sediment (Spain)	(n.d*. – 22.8)	42 (2002)
River water (Israel)	(1.8 – 3.4)	25 (2006)
Streams (USA)	831 +	23 (2002)

* n.d. Not detected. + This is a maximum value in a National Reconnaissance.

2.2.2.6: Estradiol

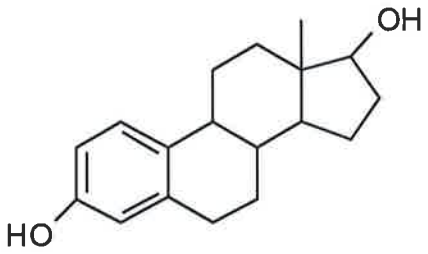


Figure 2.9: The structure of the naturally occurring estrogen, estradiol.

The structure of the estrogen, estradiol, is shown in Figure 2.9. This hormone is the major secretory product of the ovaries, being produced, in normal women, at a rate that varies from 50 to 350μg/day. Within other organs of the body it can be converted to estrone and to estriol.

Therapeutic preparations of the compound are available and these are used to treat conditions that arise from a deficiency of this hormone. Together with other estrogens it has been considered to be responsible for a variety of endocrine disruptive effects in freshwater and in marine fauna. Table 2.6 lists reported values for concentrations of the compound in different matrices.

Table 2.6: Reported values for the occurrence of estradiol in environmental matrices

Matrix	Reported values	Reference
Ocean sediment	0.9 ngg^{-1}	93
Treated effluent	14 ngL^{-1}	93
Sewage outfall	14 – 17 ngL^{-1}	91
STP effluent	< 0.8 ngL^{-1}	41
Seawater (Baltic)	< 0.3 ngL^{-1}	40
River Water	0.9 – 1.2 ngL^{-1}*	25

* This figure represents a combined value for estrone and estradiol

2.3: Collection of samples

Each site was sampled by filling three cleaned and silylised reagent bottles (2.77 L) with seawater from that site. These three bottles of seawater formed a triplicate set. The contents of each bottle were extracted and analysed separately. The water was collected in a polythene bucket and transferred immediately to the silylised amber reagent bottles fitted with teflon screw caps. As the bottles were filled completely with aqueous samples the caps were left loosely in position while the bottles were being transported to the laboratory. Screwing the caps firmly inevitably caused the bottles to break as the water expanded with the slight increase in temperature brought about by placing the bottles in a car. Prior to the collection of the samples each bottle was rinsed three times with tap water and three times with Milli-Q water. At the sampling site each bottle was rinsed with a small amount of water taken from where the samples would be collected.

Since all samples were extracted within 24 hours of collection no methods of preservation were considered necessary.

Appropriate care was taken towards personal safety when collecting all samples.

2.4: Silylisation

Silylisation is a process that deactivates the surface of glassware and thus minimises the possibility of analytes being adsorbed to that surface. It was achieved by first cleaning the glassware with aqueous detergent and then thoroughly rinsing it with water. The interior of the glass vessel was then rinsed with a 10% (v/v) solution of dichloro-dimethylsilane prepared in toluene. Finally the vessel was rinsed with toluene and then with methanol.

2.5: Sampling Campaigns

2.5.1: Samples collected from the effluent stream of the Ringsend WWTP

The effluent from the Ringsend WWTP enters Dublin bay adjacent to the Poolbeg ESB power station. The effluent joins the stream of cooling water after the latter has exited from the power station and this mixture enters the water of Dublin bay (see Plate 2.14, p.52). Six 2.77 L samples were taken from a point just downstream of the position where the effluent joins the stream of cooling water. These samples were collected and analysed in September 2006.

2.5.2: A pilot study

Prior to sampling and analysis of the complete set of fifteen randomly selected sampling sites listed in Table 2.1, p. 44 a pilot study of five of these sites was carried out. This was done to gain experience in the techniques needed to collect the samples, transport them to the laboratory and to apply methods of extraction and analysis to these samples. Though the main purpose of the pilot study was to gain experience in accessing the sampling sites and in collecting and transporting the samples the sites selected for the study were chosen to investigate the intuitive belief that, since the effluent from the WWTP is discharged on the northern side of the South Bull Wall, there would be a difference in analyte concentration in the seawater on either side of this barrier.

The sites selected for the pilot study were sites 29, 40, 70, 91 and 107 (see Figure 2.1 p.44). Collection of the samples for the pilot study was carried out in August 2006.

2.5.3: The main sampling campaign

The main sampling campaign was carried out in the autumn of 2006. Triplicate samples, labelled A, B and C, were collected from each site. Except for the samples collected from Site 91, all samples were collected within 2 hours of the high tide. All

samples were extracted within 24 hours of collection. Table 2.7 details the dates of each of the collections and the prevailing tidal conditions.

Table 2.7: Dates and prevailing tidal conditions for collection of samples for the main sampling campaign.

Sampling Site	Samples Collected	Date	Tide conditions
5	5A, 5B, 5C	05/10/06	Falling tide
19	19A, 19B, 19C	10/10/06	Rising tide
29	29A, 29B, 29C	10/10/06	Rising tide
30	30A, 30B, 30C	05/10/06	Falling tide
40	40A, 40B, 40C	03/10/06	Falling tide
50	50A, 50B 50C	03/10/06	Falling tide
70	70A, 70B, 70C	02/10/06	Rising tide
72	72A, 72B, 72C	02/10/06	Rising tide
74*	74A, 74B, 74C	11/10/06	Rising tide
76	76A, 76B 76C	09/10/06	Rising tide
82	82A, 82B 82C	11/10/06	Rising tide
91	91A, 91B, 91C	02/11/06	Low tide
107	107A, 107B, 107C	16/10/06	Falling tide
110	110A, 110B, 110C	09/10/06	Rising tide
112	112A, 112B, 112C	16/10/06	Rising tide

*A thick and extensive layer of decaying vegetation on the surface of the water prevented access to a point where clear sea water could be collected. The samples were taken from points in Site 79 as close as possible to Site 74

Chapter 3

Selection of SPE cartridges suitable for the project and design of glassware suitable for handling up to 12 samples simultaneously

3.1 Materials

The caffeine, estradiol, ethynylestradiol, ibuprofen and nonylphenol were obtained from Sigma-Aldrich. The methylparaben was from Riedel-de-Haen. The caffeine, methylparaben and ibuprofen were of 99% purity, the estradiol and ethynylestradiol were of 98% purity. The nonylphenol was technical. Methanol and acetonitrile were HPLC grade and the water was deionised using a Milli-Q system.

3.2: Solid phase extraction of water samples

Preliminary requirements of the project were to select a sorbent/cartridge that would, with satisfactory efficiency, extract the selected analytes from seawater and to build items of apparatus that would facilitate the simultaneous extraction of 12 environmental samples and the preparation, from these extracts, of samples that would be suitable for HPLC analysis.

3.3: Screening of cartridges

3.3.1: Selection of cartridges for screening

Seven different cartridges were screened for their effectiveness in extracting the analytes, caffeine, methylparaben, ethynylestradiol, ibuprofen and nonylphenol from 1 mL of a standard solution that was approximately 10 mgL^{-1} of that analyte. The cartridges screened are shown in Table 3.1.

Table 3.1: Cartridges screened for their effectiveness in extracting analytes from 1mL of a standard 10 mgL^{-1} solution

Cartridge	Dimensions
Oasis HLB (Waters)	200 mg/6 mL
Strata-X (Phenomenex)	200 mg/6 mL
Bond Elut PPL (Varian)	200 mg/6 mL
Bond Elut ENV (Varian)	200 mg/6 mL
Abselut NEXUS (Varian)	200 mg/6 mL
Strata-X-C (Phenomenex)	200 mg/3 mL
C18 Bond Elut (Varian)	500 mg/3mL

3.3.2: Conditioning and loading of cartridges

Following the procedure described by Liu *et al.* [91] each cartridge was conditioned with 5 mL of ethyl acetate, followed by 5 mL of methanol followed by 5 mL of water. After loading, the sorbent was washed with 1 mL of water and then the adsorbed analyte was eluted with 2 mL of ethyl acetate. The extract was then blown down almost to dryness with nitrogen gas and reconstituted with 1 mL of acetonitrile. These extracts were then analysed by HPLC with UV detection simultaneously with the loading solution.

3.3.3: HPLC analysis of the extracts

The extracts were analysed by HPLC using a mobile phase of 45% Acetonitrile/0.2%triethylamine$_{(aq)}$ solution adjusted to pH 3.2 with orthophosphoric acid. The injection volume was 20 μL and detection of the analytes was at 220 nm. Each sample was injected three times and the mean value was used in subsequent calculations. A value for the % recovery was estimated by comparison of the mean peak area of the extract, with the mean area of the standard solution that had been used to load the cartridges, each

sample being injected three times. The results (mean peak areas and % recoveries) are listed in Table 3.2(d) – (e) and shown graphically in Figure 3.1.

Table 3.2(a): Mean peak areas and % recovery of caffeine using different cartridges

	Mean peak area (n = 3 injections)	% Recovery
Standard Solution Caffeine	535.4	100
Oasis HLB	518.3	96.8
Strata-X	570.1	106.5
Bond Elut PPL	345.1	64.5
Bond Elut ENV	336.3	62.8
Abselut NEXUS	601.9	112.4
Strata-X-C	233	43.5
C18	439.2	82

Table 3.2(b): Mean peak areas and % recovery of methylparaben using different cartridges

	Mean peak area (n = 3 injections)	% Recovery
Standard Solution Methylparaben	915.9	100
Oasis HLB	878.2	95.9
Strata-X	897.4	98
Bond Elut PPL	339.8	37.1
Bond Elut ENV	484.1	52.9
Abselut NEXUS	944.2	103.1
Strata-X-C	992.6	108.4
C18	1067.8	116.6

Table 3.2(c): Mean peak areas and % recovery of ethynylestradiol using different cartridges

	Mean peak area (n = 3 injections)	% Recovery
Standard Solution Ethynylestradiol	228.4	100
Oasis HLB	194.7	85.2
Strata-X	206.9	90.6
Bond Elut PPL	130.6	57.2
Bond Elut ENV	150.3	65.8
Abselut NEXUS	233.1	102.1
Strata-X-C	218.1	95.5
C18	258.4	113.1

Table 3.2(d): Mean peak areas and % recovery of ibuprofen using different cartridges

	Mean peak area (n = 3 injections)	% Recovery
Standard Solution Ibuprofen	405	100
Oasis HLB	414.9	102.4
Strata-X	347.3	85.8
Bond Elut PPL	116	28.6
Bond Elut ENV	335.5	82.8
Abselut NEXUS	111.5	27.5
Strata-X-C	353.1	87.2
C18	321.6	79.4

Table 3.2(e): Mean peak areas and % recovery of nonylphenol using different cartridges

	Mean peak area (n = 3 injections)	% Recovery
Standard solution Nonylphenol	242.35	100
Oasis HLB	160.6	66.3
Strata-X	157	64.8
Bond Elut PPL	49.7	20.5
Bond Elut ENV	144.5	59.6
Abselut NEXUS	148.5	61.3
Strata-X-C	92.7	38.3
C18	125.2	51.7

The information listed in Tables 3.2(a) to 3.2(e) is shown graphically in Figure 3.1

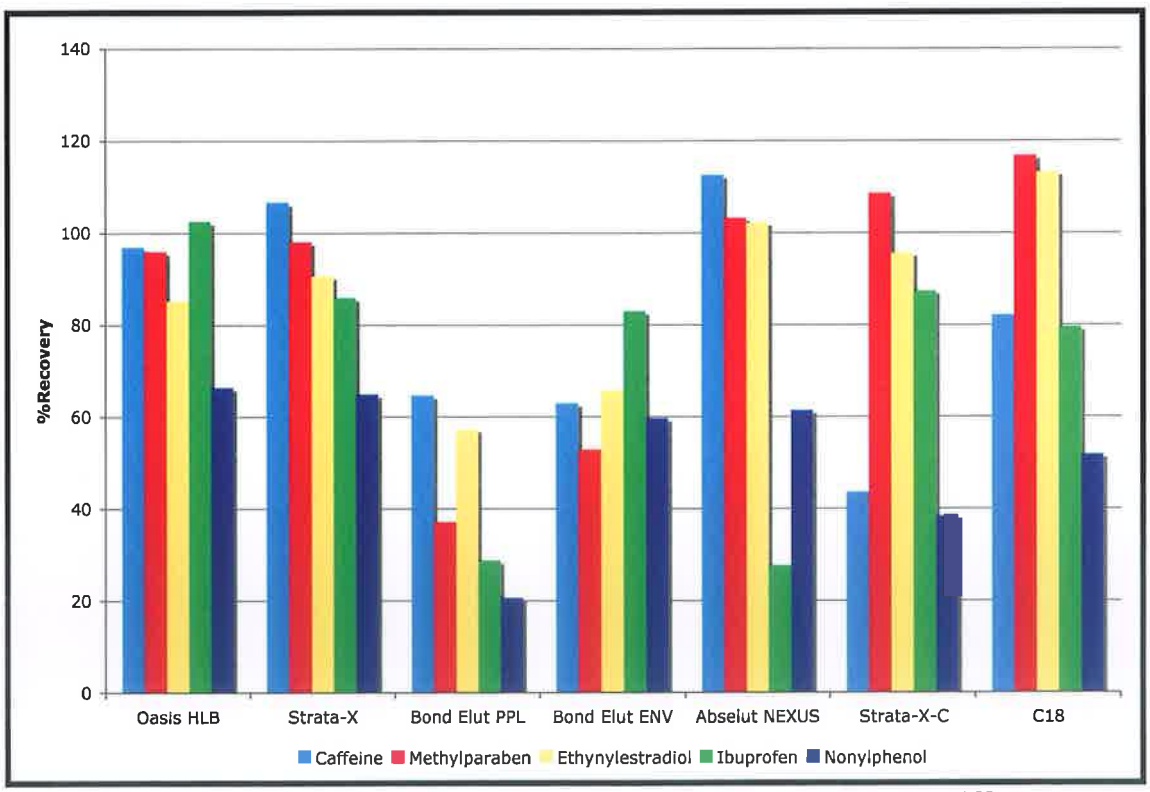


Figure 3.1: Histogram showing the comparative effectiveness of seven different cartridges in extracting an analyte from 1 mL of a standard (10 mgL^{-1}) solution

3.3.4: Choice of cartridge/sorbent for the project

The results summarised in Figure 3.1 indicate that the cartridges Oasis HLB, Strata-X, Abselut NEXUS and Varian C18 perform well when extracting the analytes from standard solutions. However, preliminary work had shown, previously, that Varian C18 cartridges were unsuitable for work with environmental samples since this sorbent readily became blocked with an accumulation of the suspended matter that is inevitably found in such samples.

3.3.5: The cost of cartridges

The cartridges Oasis HLB, Strata-X and Abselut NEXUS were considered suitable for the extraction of the analytes of interest. Oasis HLB and Strata-X were significantly less expensive than NEXUS so these were considered for further work. Comparative cost of the cartridges is shown in Table 3.3.

Table 3.3: Comparative cost of cartridges

Cartridge/Sorbent	Cost/30 cartridges (Euros)
Oasis HLB	135.00
Strata-X	108.00
Abselut NEXUS	188.60

3.4: Preparation and use of synthetic seawater

3.4.1: Preparation of synthetic seawater

Following the method described by Fiol *et al.* [94], 20 L of synthetic seawater (SSW) were prepared. Table 3.4 shows the recipe for the SSW.

Table 3.4: "Recipe" for SSW as described by Fiol

Component	$Molkg^{-1}$
NaCl	0.42664
KCl	0.01058
$CaCl_2$	0.01077
$MgCl_2$	0.05518
Na_2SO_4	0.02926

The amounts of the different components used in the preparation of 20 L of SSW are shown in Table 3.5.

Table 3.5: Amounts of components used to prepare 20 L of SSW

Component	$Mol(20kg)^{-1}$	$M_R\ (gmol^{-1})$	$g(20kg)^{-1}$
NaCl	8.5328	58.44	498.7
KCl	0.2116	74.55	15.8
$CaCl_2$	0.2154	110.98	23.9
$MgCl_2$	1.1036	95.21	105.1
Na_2SO_4	0.5852	142.04	83.12

3.4.2: Comparison of the effectiveness of two sorbents using synthetic seawater (SSW)

The effectiveness of Strata-X (Phenomenex) and of Oasis HLB cartridges in extracting the analytes, caffeine, methylparaben, ethynylestradiol and ibuprofen from this SSW was determined. The procedure outlined in Table 3.6 was carried out with Strata-X and with Oasis HLB cartridges.

Table 3.6: Procedure for the estimation of the comparative effectiveness of Strata-X and Oasis HLB cartridges in extracting analytes from SSW

2 L Aliquot of SSW	Treatment
A	Not Spiked
B	Not Spiked
C	Spiked with 1 mL of a solution containing 10 mg L^{-1} of each of the four analytes.
D	Spiked with 1 mL of a solution containing 10 mg L^{-1} of each of the four analytes

Each of A, B, C and D was extracted with a cartridge that had been conditioned with 5 mL of methanol followed by 5 mL of water. Loading of the cartridges took a little over 6 hours which equates to about 6 $mLmin^{-1}$. After loading, each cartridge was washed with 1 mL of water and then eluted with 6 mL of acetonitrile. These extracts were then blown down to about 100 µL with nitrogen gas and then reconstituted with 1 mL of 50% acetonitrile.

3.4.3: Analytical method

Using HPLC they were analysed by comparison with the solution of standards that had been used to spike the SSW. Each measurement used was the mean of three injections. The following HPLC method of analysis was used: A mobile phase of 50%

Acetonitrile/0.2%Triethylamine (TEA) solution adjusted to pH 3.2 with orthophosphoric acid at a flow rate of 1 $mLmin^{-1}$, 20 μL injection volume, detection at 220 nm.

A % recovery was estimated by averaging the peak areas of the extracts of C and of D and expressing this as a % of the peak area of the standard solution. The results for each type of cartridge are shown in Tables 3.7(a) and 3.7(b).

Table 3.7(a): Peak areas for the standard solution and for the extracts using Strata-X cartridges

	Standard solution ($10\ mgL^{-1}$)	Extract from Cartridge C	Extract from Cartridge D	% Recovery (Mean)
Caffeine	594.4	409.7	434.5	71
Methylparaben	441.0	424.0	465.9	101
Ethynylestradiol	237.1	231.2	230.6	97.4
Ibuprofen	353.0	134.5	192.6	46.3

Table 3.7(b): Peak Areas for the standard solutions and for the extracts using Oasis HLB cartridges

	Standard solution ($10\ mgL^{-1}$)	Extract from Cartridge C	Extract from Cartridge D	% Recovery (Mean)
Caffeine	585.3	451	466.2	78.4
Methylparaben	416.3	379.5	363.2	89.2
Ethynylestradiol	211.3	158.8	147.4	72.5
Ibuprofen	316	38.2	14.8	8.4

The information listed in Tables 3.7(a) and 3.7(b) is shown graphically in Figures 3.2 and 3.3.

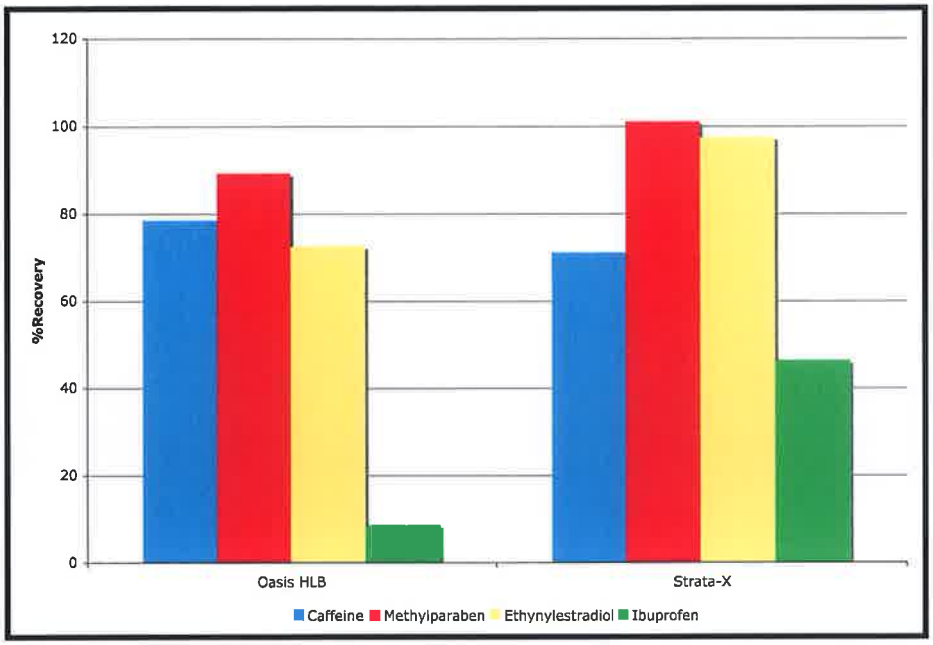


Figure 3.2: Comparison of the % recovery of analytes from spiked synthetic seawater using different cartridges. Method of recovery described in section 3.3.3

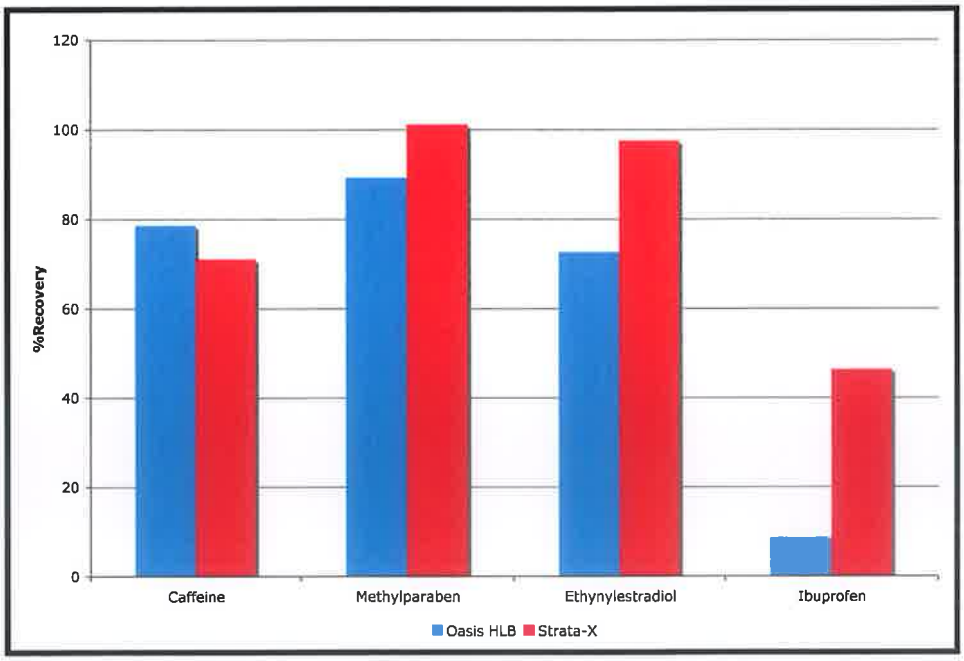


Figure 3.3: Comparison of the % recovery of analytes from spiked synthetic seawater using different cartridges. Method of recovery described in section 3.3.3

3.4.4: Selection of cartridge for project

Exclusive of ibuprofen, for which neither sorbent gave a satisfactory recovery, the mean recovery of the analytes was 89.9% using Strata-X and 80% using Oasis-HLB. The former was selected for the project.

3.5: Acidification of environmental samples prior to extraction

Established methods for the extraction of acidic drugs from environmental samples by SPE frequently include acidification of the sample prior to extraction. This procedure suppresses ionization of the acidic analyte and thus facilitates adsorption of the molecular form by the reversed phase sorbent.

The acidification procedure was tested by comparing the chromatograms of extracts of acidified and non-acidified samples of WWTP effluent. These two extracts were analysed by injecting 20 μL of each extract into a mobile phase of 20% acetonitrile at a flow rate of 0.8 $mLmin^{-1}$ and monitored at 272 nm. The respective chromatograms are shown in Figures 3.4(a) and 3.4(b).

The occurrence of a broad, undifferentiated peak in the chromatogram of the extract of the acidified sample (Figure 3.4(a)) indicates that this step is not suitable for analytes that would elute in this region. This situation contrasts with that where the sample has not been acidified prior to extraction (Figure 3.4(b)).

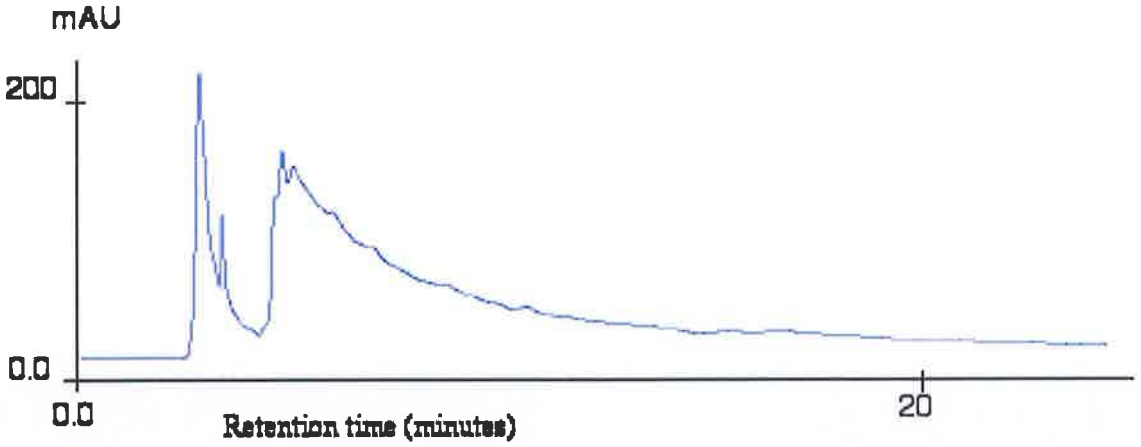


Figure 3.4(a): Chromatogram of WWTP extract that had been acidified to pH 3 with concentrated sulphuric acid prior to extraction. 20µL extract, separated with 20 % acetonitrile at 0.8 mLmin^{-1} and monitored at 272 nm.

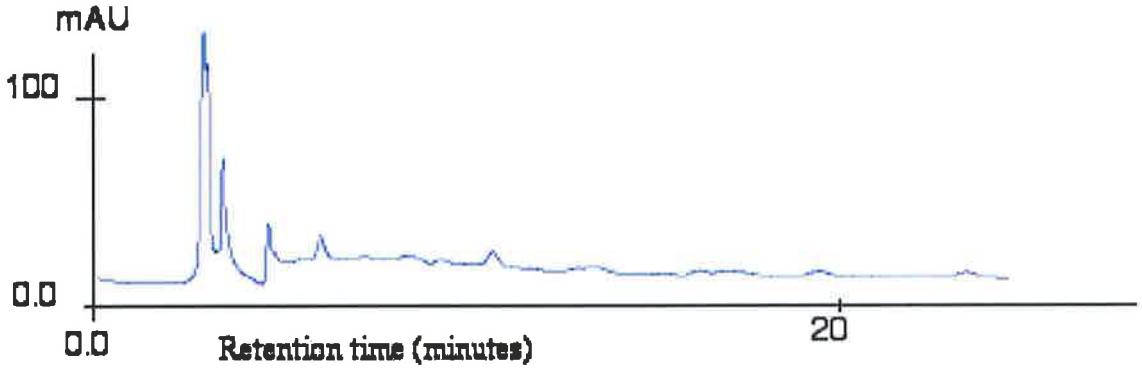


Figure 3.4(b): Chromatogram of WWTP extract that had not been acidified prior to extraction. 20µL extract, separated with 20 % acetonitrile at 0.8 mLmin^{-1} and monitored at 272 nm.

3.6: Wash of the sorbent after extraction

The purpose of washing the sorbent prior to elution of the retained analytes is to remove as much interfering material as possible without removing the target analytes. Varying concentrations of aqueous methanol were tested for this purpose. This was done by spiking each of five 1L aliquots of seawater with 1mL of a 2 mgL^{-1} methanolic solution of caffeine and methylparaben and extracting them as described. After loading the cartridges the sorbents were separately washed with 3 mL of 0%, 5%, 10%, 20% and 30% aqueous methanol. The cartridges were then dried and the retained analytes were eluted as described. These extracts were analysed. Two selected chromatograms are shown in Figures 3.5(a) and 3.5(b).

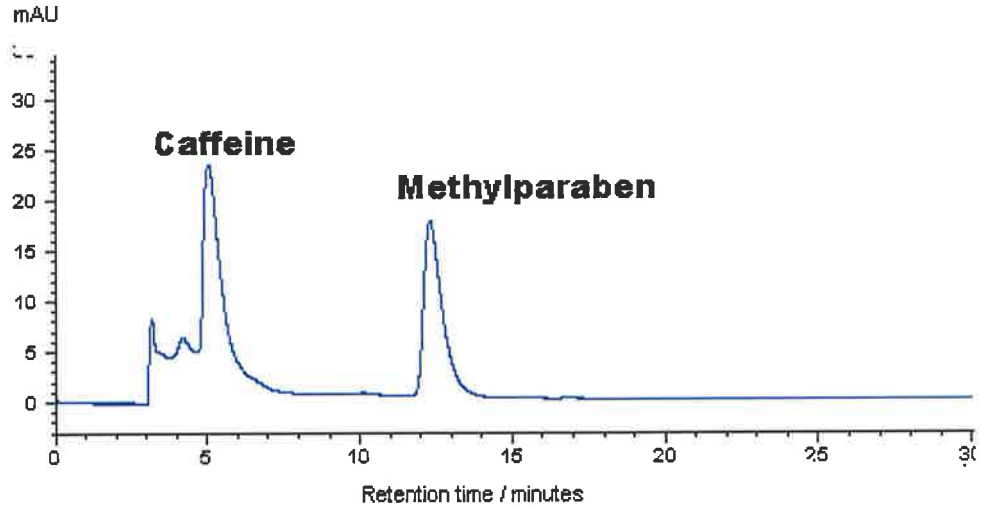


Figure 3.5(a): Chromatogram of extract of 1 L seawater spiked with 1 mL 2 mgL^{-1} caffeine and methylparaben after washing sorbent with 3 mL water prior to elution. Detection at 272 nm.

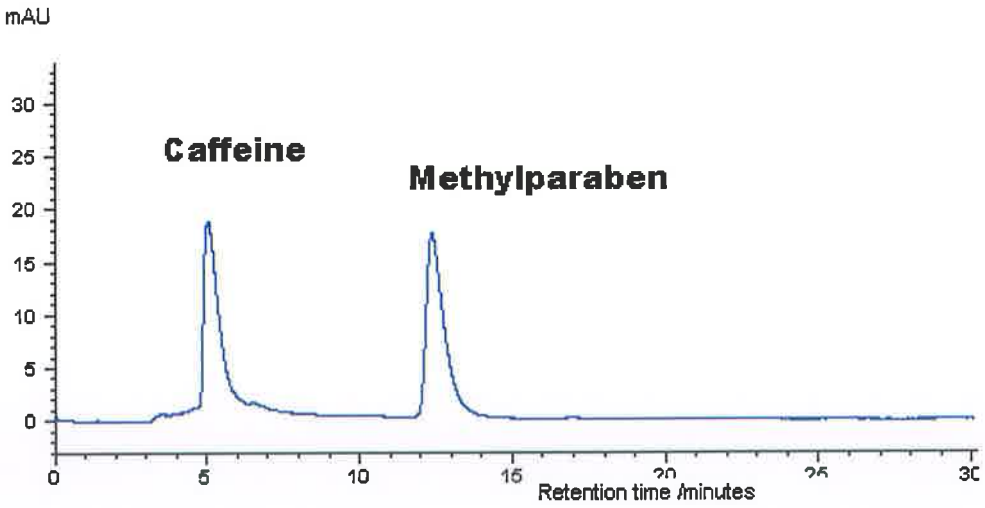


Figure 3.5(b): Chromatogram of extract of 1 L seawater spiked with 1 mL 2 mgL^{-1} caffeine and methylparaben after washing sorbent with 3 mL 30% methanol prior to elution. Detection at 272 nm.

As can be seen from these chromatograms a wash of 30% methanol has the effect of removing an appreciable amount of the interference that occurs in seawater without,

apparently, diminishing the concentration of the target analytes to any great extent. However, when this same wash was used with concentrations of the analytes comparable to the concentrations that are likely to occur in environmental samples the analytes were removed completely. Thus a wash of water alone used.

3.7: SPE method for the extraction of analytes from seawater

The following method was considered appropriate for the extraction of the analytes from a 2.77L reagent bottle full of seawater (for the % recovery of the analytes by this method see below Ch 4)

Table 3.8: Solid phase extraction (SPE) method

SPE cartridge	Strata-X (Phenomenex) (200 mg, 6 mL)
Flow of solution through cartridge	6 $mLmin^{-1}$
Drying	15 min
Elution of adsorbed analytes	6 (3×2) mL acetonitrile
Treatment of eluate	Reduced to ~50 μL under $N_{2(g)}$ and then reconstituted to 1 mL with methanol

3.8: Glassware designed and fabricated for the project

3.8.1: A 'homemade' vacuum manifold

The project required the extraction of triplicate samples from 15 sampling sites within as short a period of time as possible. Since the extraction of each 2 L sample takes about 6 hours it was essential to gain access to apparatus that would allow the simultaneous extraction of several samples. Manifolds designed for this purpose are available commercially, however, owing to the expense of such equipment apparatus that could be made by a glassblower was designed. The apparatus is shown in Figure 3.6. It allowed for the simultaneous extraction of up to 12 samples. A photograph of this apparatus is shown in Plate 3.1.

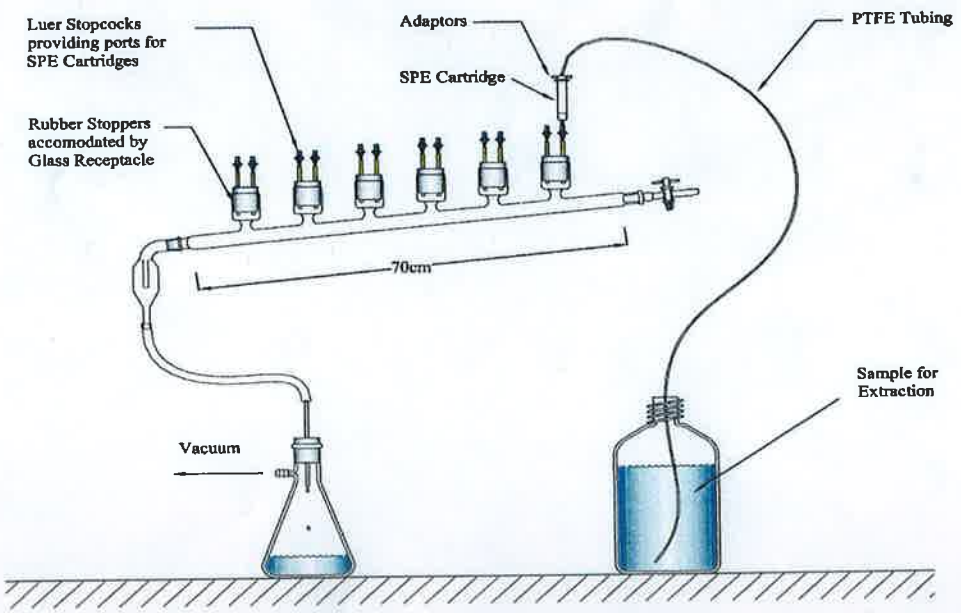


Figure 3.6: Glass manifold designed and built to facilitate the simultaneous extraction of 12 samples. The flask attached to the vacuum pump shown is a 2 L Buchner flask. In practice a 10 L flask was used.

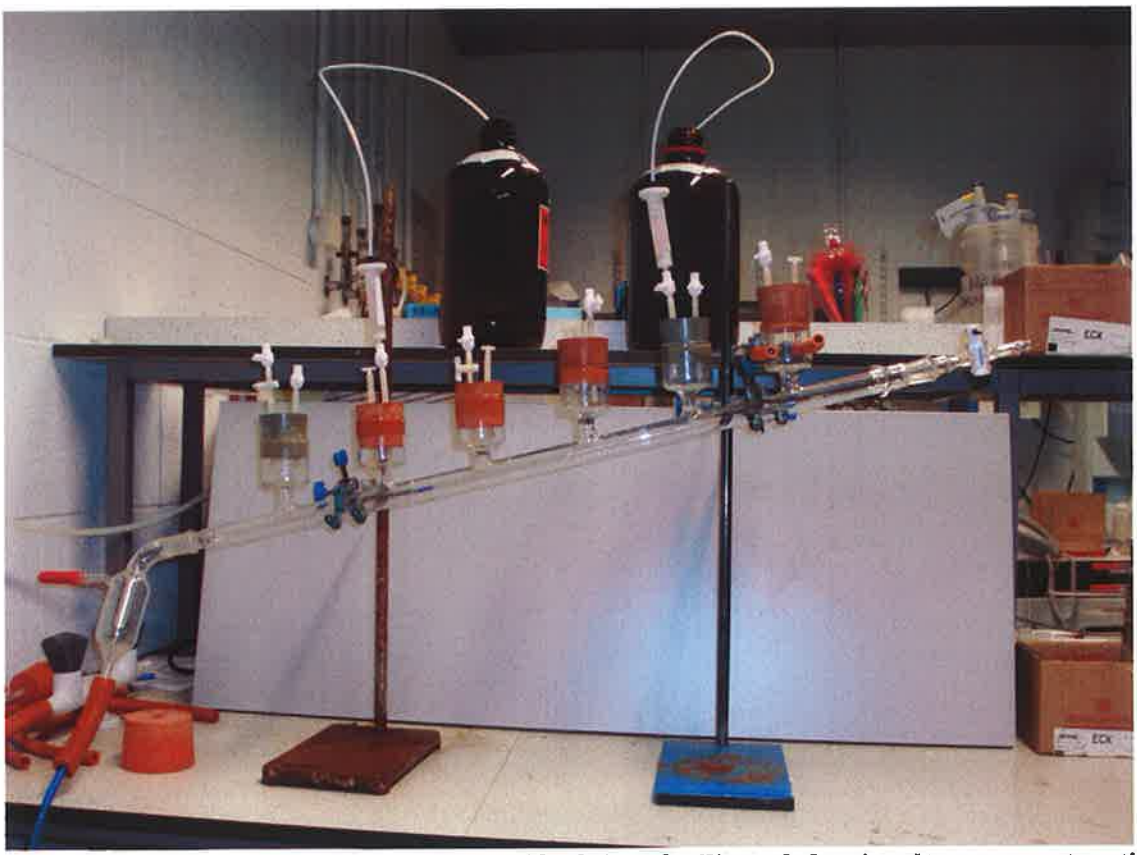


Plate 3.1: Photograph of the glass manifold that facilitated the simultaneous extraction of up to 12 environmental samples. The 10 L evacuated flask in which the excess extracted sample collected is not shown.

3.8.2: Simultaneous evaporation of solvent from 12 extracts

The volume of the extracts produced by the SPE method (Table 3.8) was 6 mL. To convert these into 1 mL samples suitable for HPLC analysis it was necessary to evaporate excess solvent. A second piece of glassware that would allow the simultaneous evaporation of solvent from 12 samples by means of a flow of nitrogen was designed. This second item is shown diagrammatically in Figure 3.7. A photograph of this apparatus is shown in Plate 3.2.

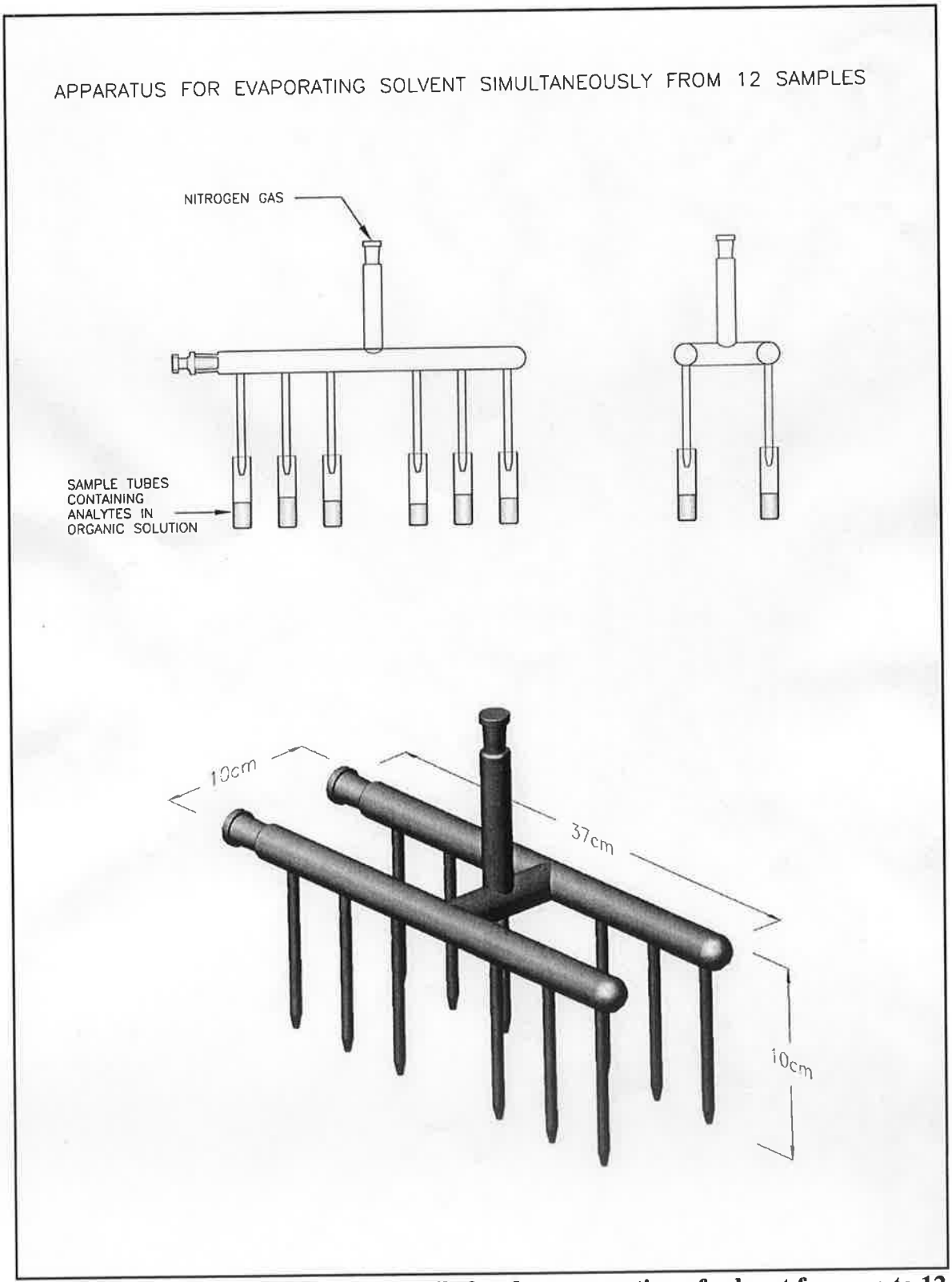


Figure 3.7: Glassware designed and built for the evaporation of solvent from up to 12 samples by means of a flow of nitrogen gas.

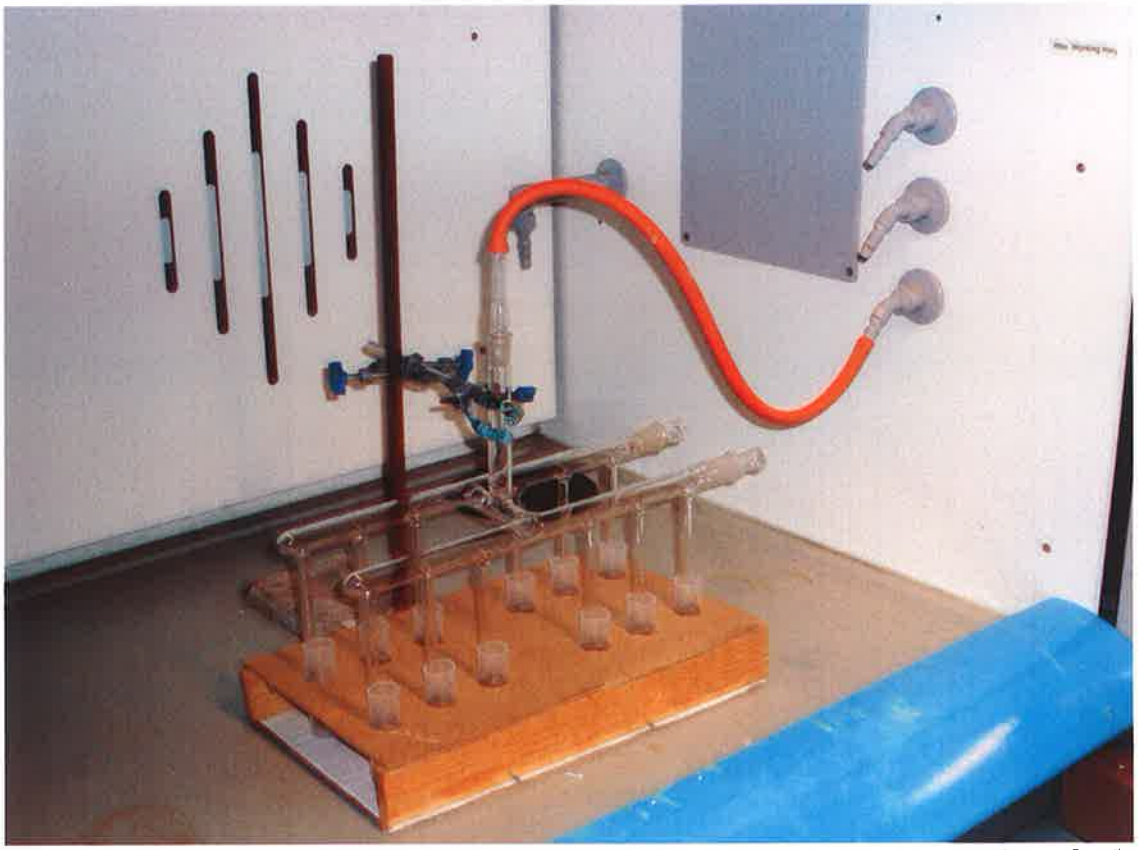


Plate 3.2: Photograph of glassware that allowed the simultaneous evaporation of solvent from 12 samples by means of a flow of nitrogen gas.

Chapter 4

Development of analytical separation methods and estimation of the % recovery of the SPE method.

4.1: Introduction

Successful detection and accurate quantification of analytes by means of HPLC is dependent upon achieving chromatograms in which the peaks are adequately resolved and of satisfactory height with respect to the background noise. When such has been achieved it is then necessary to establish minimum values of analyte concentration below which detection cannot be established and a minimum level below which the analyte cannot be accurately quantified. A concentration interval within which there is a linear relationship between the signal, i.e. the peak height or area, and the analyte concentration must also be established and this relationship should be expressed in the form of a linear calibration equation. Once such HPLC methods have been established it is possible to combine them with an established SPE method to give an overall method for the extraction, detection and the quantification of the analytes in water. A %recovery of the analytes from synthetic seawater (SSW) can then be estimated by spiking known volumes of SSW with standard solutions of the analytes, extracting the analytes from these known volumes, analysing these extracts and comparing the results of this analysis with the known quantity initially spiked into the SSW.

4.2: Separation and detection of the analytes

4.2.1: Liquid chromatographic separation of analytes

For each of the analytes other than ibuprofen the pK_a value is sufficiently high for analysis to be carried out using a neutral mobile phase. The pK_a value of ibuprofen is such that a buffered mobile phase is necessary. A value that has been suggested for the pK_a of ibuprofen is 5. In a neutral solution this compound will be almost completely ionised and therefore will not be retained by a reversed phase column. A mobile phase buffered to about pH 3 is needed to ensure that ibuprofen exists predominantly in the neutral form. Aqueous acetonitrile adjusted to pH 3.2 with triethylamine (TEA) and orthophosphoric acid (H_3PO_4) as described by Ravisankar *et al.* [95] was used to demonstrate the influence of decreasing organic content in the mobile phase on the

retention time of the analytes in the course of an isocratic separation. This work was carried out on a Hewlett Packard 1050 HPLC system equipped with an autosampler using a Varian, C18, 250×046, 5 μm column. In each case 20 μl of samples that were 100 mgL^{-1} for each of the analytes caffeine, methylparaben, ethynylestradiol, ibuprofen and nonylphenol were injected into the mobile phase flowing at 1 $mLmin^{-1}$ and the analytes were detected at 220 nm. Two chromatograms are shown, Figures 4.1 and 4.2.
The results are shown graphically in Figure 4.3.

Discussion

The chromatograms shown in Figures 4.1 and 4.2 illustrate the effect of decreasing organic content in the mobile phase on the retention time of the analytes. With 70% acetonitrile caffeine and methylparaben are not resolved though ethynylestradiol, ibuprofen and nonylphenol are well separated within a convenient time interval. Significant, but incomplete, resolution of caffeine and methylparaben is achieved with 55% acetonitrile but the retention time of nonylphenol has increased conciderably and its peak has become considerably broader.

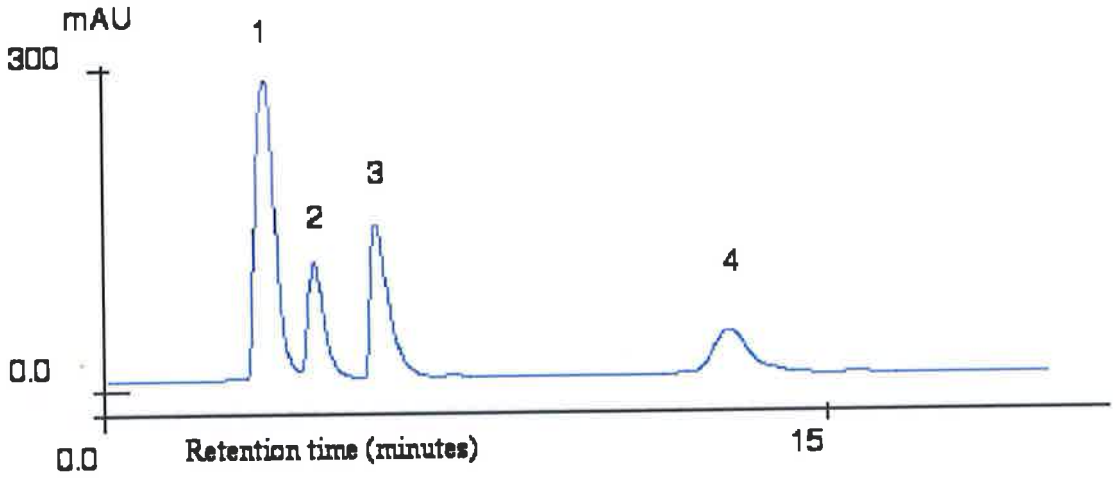


Figure 4.1: Separation of analytes using 70% acetonitrile/0.2% TEA solution at pH 3.2, detection at 220 nm.. 1 = caffeine, methylparaben (not resolved), 2 = ethynylestradiol, 3 = ibuprofen, 4 = nonylphenol

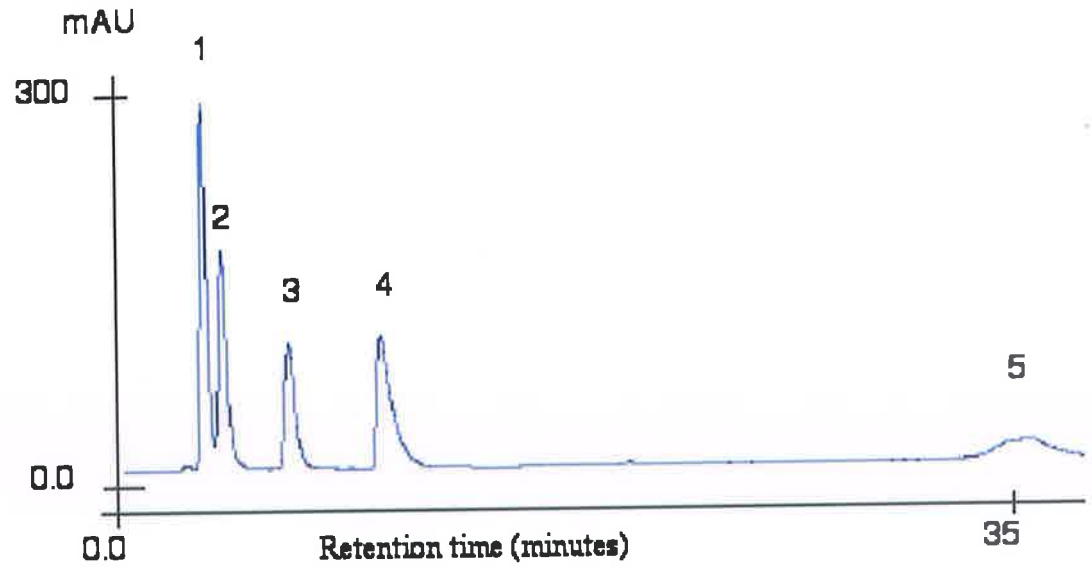
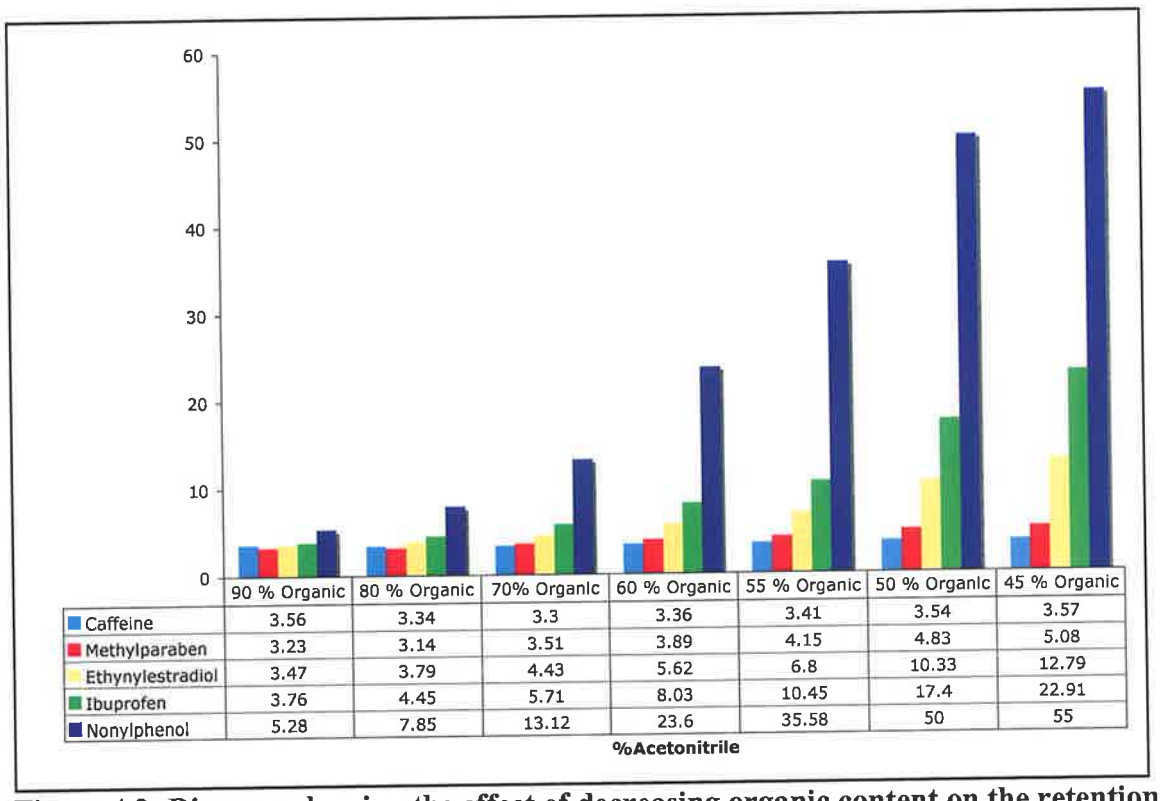


Figure 4.2: Separation of analytes using 55% acetonitrile/0.2% TEA solution pH 3.2, detection at 220 nm.. 1 = caffeine, 2 = methylparaben, 3 = ethynylestradiol, 4 = ibuprofen, 5 = nonylphenol



	90 % Organic	80 % Organic	70% Organic	60 % Organic	55 % Organic	50 % Organic	45 % Organic
Caffeine	3.56	3.34	3.3	3.36	3.41	3.54	3.57
Methylparaben	3.23	3.14	3.51	3.89	4.15	4.83	5.08
Ethynylestradiol	3.47	3.79	4.43	5.62	6.8	10.33	12.79
Ibuprofen	3.76	4.45	5.71	8.03	10.45	17.4	22.91
Nonylphenol	5.28	7.85	13.12	23.6	35.58	50	55

Figure 4.3: Diagram showing the effect of decreasing organic content on the retention time of five analytes. For 50% and 55% acetonitrile retention times of 50 and 55 minutes have been inserted. In fact in these cases this compound did not elute.

4.2.2: Methods of separation suitable for environmental samples

As Figure 4.2 suggests the analytes can be separated isocratically using 55% acetonitrile, However, this method is unsuitable for the analysis of environmental extracts since the short retention time of caffeine causes its signal to be inevitably obscured by the broad peak arising from humic material that is frequently found in such samples. Furthermore, with this mobile phase the retention time of the more hydrophobic nonylphenol is long and its peak is somewhat too broad for accurate quantification. Thus, three separate mobile phases of different organic content were used to analyse the extracts of the environmental samples. This ensured that each of the analytes could be detected within a convenient time interval. The buffered mobile phase described above was used for the analysis of ibuprofen, otherwise no buffer was used. The methods are summarised in Table 4.2.

4.2.3: Methods of detection

Two methods of detection were employed, UV-absorbance and, where appropriate, natural fluorescence of the analyte molecules. Estimation of the analyte concentration, in both cases, is dependent upon a linear relationship between the concentration of the analyte and the intensity of the signal.

4.2.4: Fluorescence detection (FLD)

Since estrogens manifest natural fluorescence this method of detection was used for the analysis of these compounds. Two reported methods of fluorescence detection were compared. In each case separation was achieved using 40% acetonitrile at a flow rate of 1 $mLmin^{-1}$. In each case 20 µL of a 10 mgL^{-1} solution was injected. The results are shown in Table 4.1

Table 4.1: Peak areas obtained when 20 µL of a 10 mgL^{-1} solution were separated and detected by two different methods

Ex/Em (nm)	Reference	Estriol	Estradiol	Ethynylestradiol
280/312	96	865.2	972.2	916.5
230/310	97	1354.2	1556.2	1526.4

Excitation at 230 nm and emission at 310 nm gives a significantly greater peak area for each of the estrogens. Accordingly this was selected as the more effective method of detection. Table 4.2 lists the methods employed for the detection of analytes using UV-detection. Table 4.3 lists the methods employed using FLD.

Table 4.2: HPLC methods with UV-detection

Analyte	Mobile Phase (% acetonitrile)	Rate of Flow (mL/minute)	Injection volume (μL)	Wavelength of detection (nm)
Caffeine	20	0.8	20	272
Methylparaben	45	1.0	20	254
and Ethynylestradiol				220
Ibuprofen	70	1.0	20	220
and Nonylphenol	(pH 3.2 with TEA and H_3PO_4)			220

Table 4.3: HPLC methods with FLD

Analyte(s)	Mobile phase (%Acetonitrile)	Rate of Flow ($mLmin^{-1}$)	Injection Volume (μL)	Ex/Em (nm)
Ethynylestradiol and Estradiol	45	1.0	20	230/310

4.2.5: Calibration curves and limits of detection and of quantification

Calibration curves, equations and limits of detection (LOD) and quantification (LOQ) were established for each of the methods. The LOD was that analyte concentration that gave a signal to noise ratio of three; the LOQ was that which gave a ratio of ten. In the case of caffeine the curve and limits were established by taking the mean of five injections of appropriate standard solutions. For each of the other analytes the mean values of three injections were employed.

The calibration equation for caffeine with confidence limits is shown in Figure 4.4. The calibration results for each of the methods are shown in Tables 4.4 and 4.5.

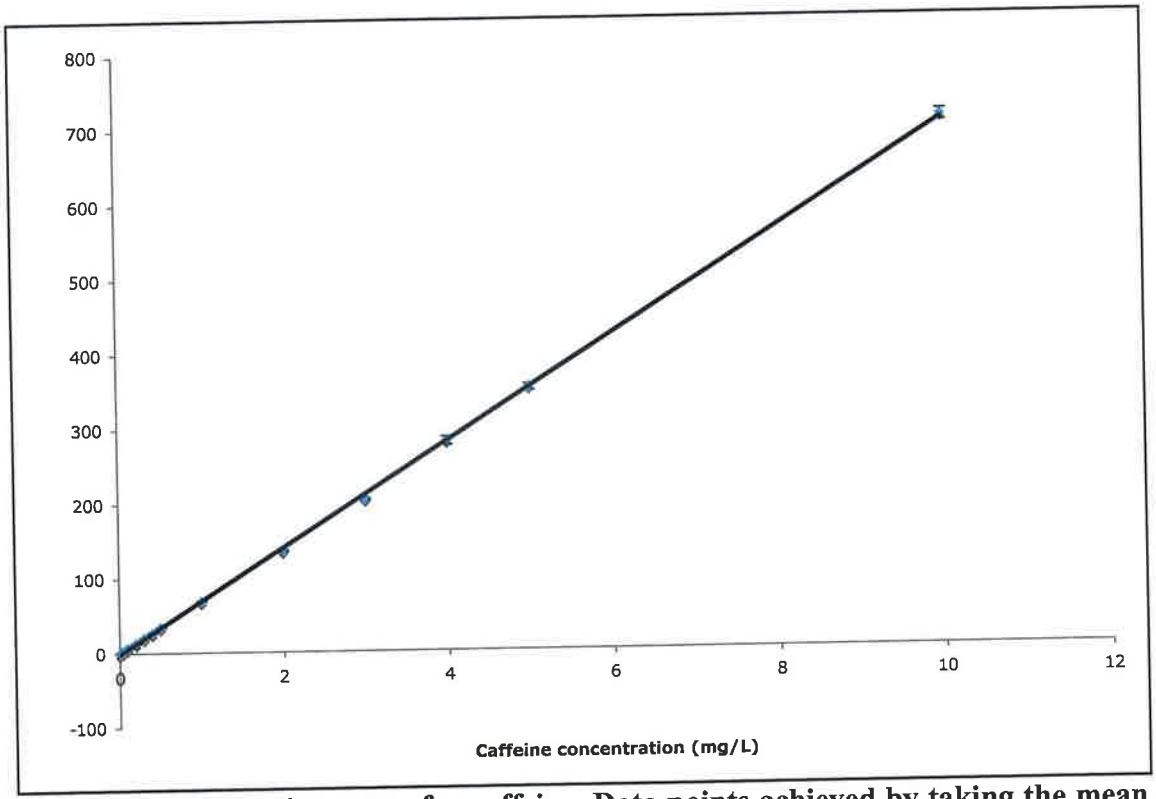


Figure 4.4: Calibration curve for caffeine. Data points achieved by taking the mean area from five injections. Error bars represent standard deviations. Calibration equation, $Y = 70.8X - 1.3216$, $R^2 = 0.9998$

Table 4.4: Calibration results for HPLC methods with UV-detection

Analyte	Calibration equation	Limit of detection(LOD) (mg/L)	Limit of Quantification(LOQ) (mg/L)
Caffeine	$Y = 70.8X - 1.3216$ $R^2 = 0.9998$	0.005	$0.02 < X < 10$
Methylparaben	$Y = 106.84X - 1.394$ $R^2 = 0.9999$	0.0025	$0.01 < X < 10$
Ethynylestradiol	$Y = 27.51X - 0.1424$ $R^2 = 1$	0.025	$0.05 < x < 0.75$
Ibuprofen	$Y = 43.525X + 0.3842$ $R^2 = 0.9999$	0.025	$0.05 < X < 10$
Nonylphenol	$Y = 22.976X - 3.6567$ $R^2 = 0.9996$	0.1	$0.5 < X < 5$

Table 4.5: Calibration results for HPLC methods with FLD

Analyte	Calibration equation	LOD (mg/L)	LOQ (mg/L)
Ethynylestradiol	$Y = 144.04X + 1.241$ $R^2 = 1$	0.005	$0.0075 < X < 10$
Estradiol	$Y = 148.6X + 1.4613$ $R^2 = 1$	0.0025	$0.05 < X < 10$

All methods were linear within the specified concentration ranges. As indicated above detection by means of fluorescence is expected to be more sensitive than by means of absorbance. The lower LOD achieved using FLD is in accordance with this expectation.

4.2.6: Chromatograms

Chromatograms at the limit of detection of the analytes with UV-detection are shown in Figures 4.5 to 4.9 and with FLD in Figures 4.10 and 4.11.

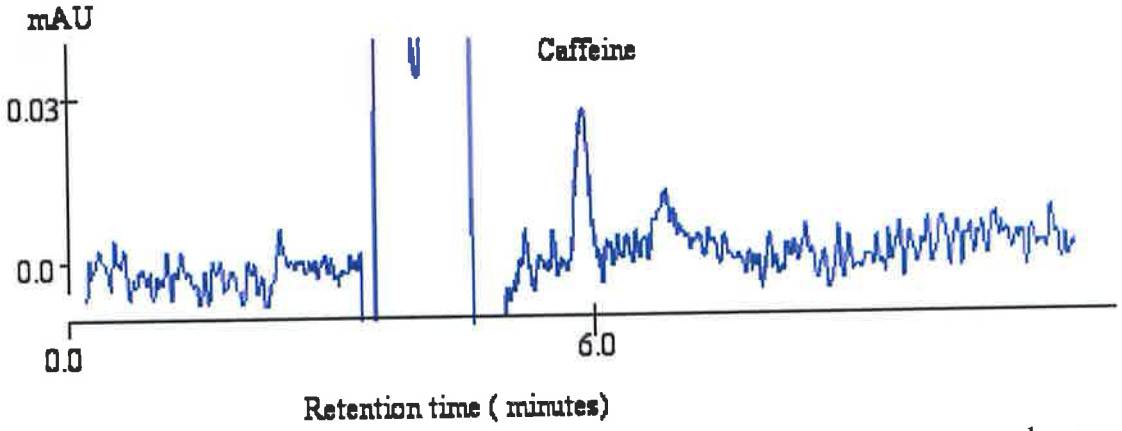


Figure 4.5: Retention time of caffeine 6 minutes with 20% acetonitrile at 0.8 mLmin^{-1}. LOD 0.005 mgL^{-1}. Detection at 272 nm.

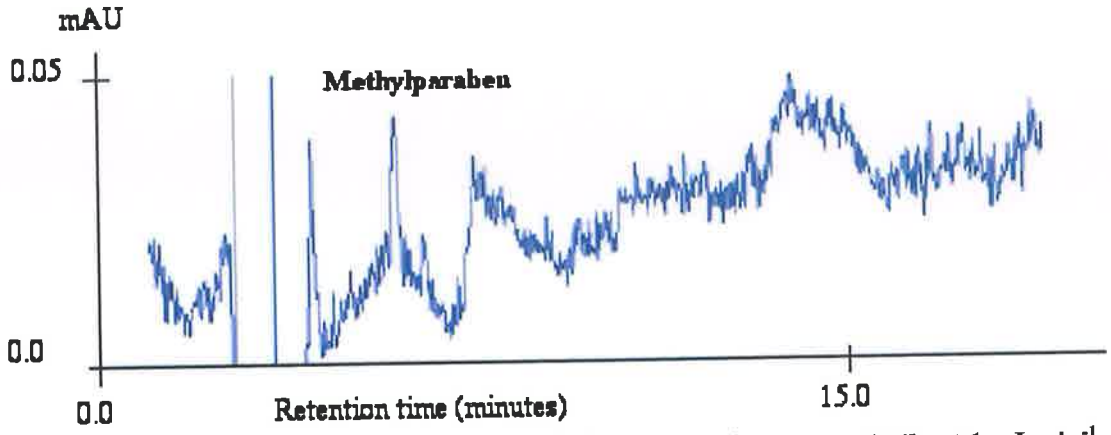


Figure 4.6: Retention time of methylparaben 5.7 mins with 45% acetonitrile at 1 mLmin^{-1}. LOD 0.0025 mgL^{-1}. Detection at 254 nm.

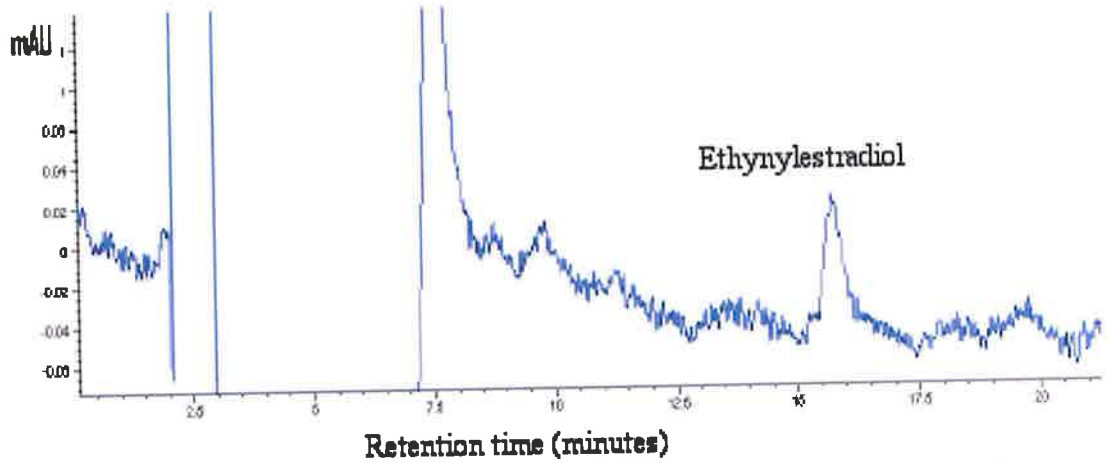


Figure 4.7: Retention time of ethynylestradiol 16.1 min with 45%acetonitrile at 1 mLmin^{-1}. LOD 0.025 mgL^{-1}. Detection at 220nm

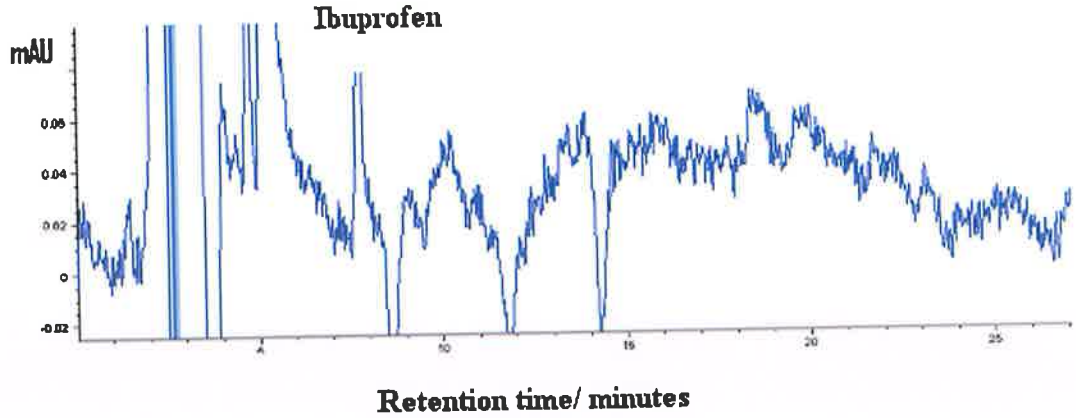


Figure 4.8: Retention time of ibuprofen 7.7 min with 70% acetonitrile (pH 3.2) at 1 mLmin^{-1}. LOD 0.025 mgL^{-1}. Detection at 220 nm.

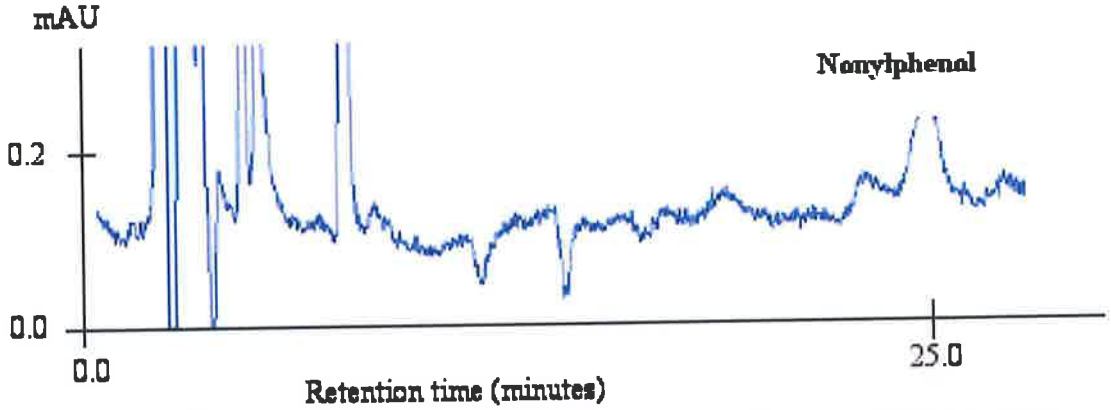


Figure 4.9: Retention time of nonylphenol 25 min with 70% acetonitrile (pH 3.2) at 1 mLmin^{-1}. LOD 0.1 mgL^{-1}. Detection at 220 nm.

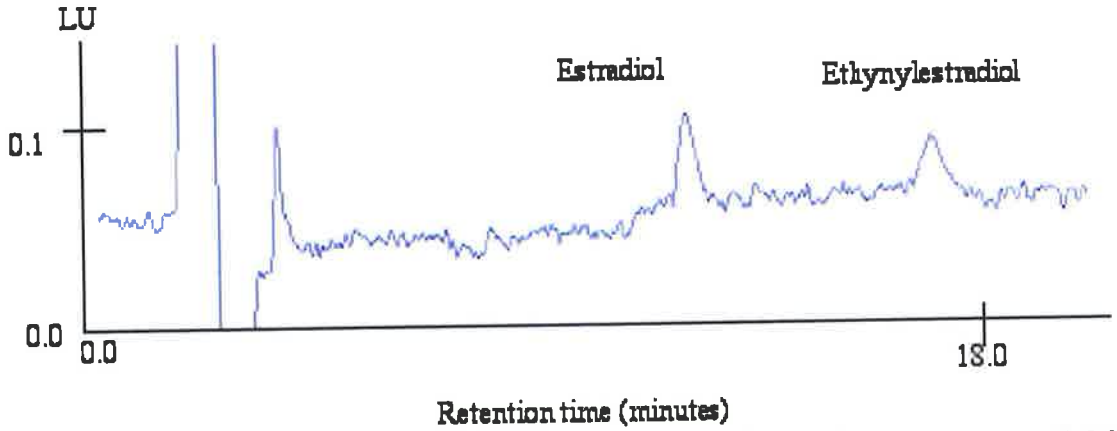


Figure 4.10: Retention time ethynylestradiol, 16.6 mins. LOD Ethynylestradiol 0.005 mgL^{-1}. FLD at Ex 230nm, Em 310nm.

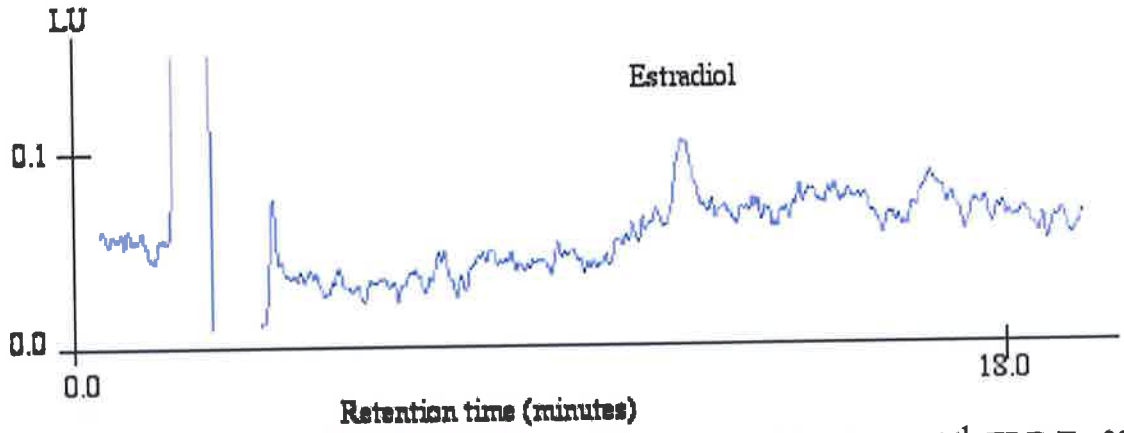


Figure 4.11: Retention time of estradiol 11.8 minutes. LOD 0.0025 mgL^{-1}. FLD Ex 230 nm, Em 310 nm.

4.3: Estimation of % recovery of method

A statistically robust estimate of the % recovery of the analytes from SSW using the SPE method described in Section 3.6 and the HPLC methods of analysis summarized in Tables 4.2 and 4.3 was established by considering the mean of five extractions. This was done by taking five reagent bottles filled with synthetic seawater and spiking each with one mL of a methanolic solution that was 10 mgL^{-1} for each of the analytes, caffeine, methylparaben, ethynylestradiol, estradiol, and nonylphenol and 20 mgL^{-1} for ibuprofen. The contents of each bottle were extracted using the established SPE method and the 1 mL extracts were then analysed for each of the analytes using the established HPLC methods. These extracts together with the loading solution, i.e. the solution used to spike the SSW volumes, were analysed simultaneously with two standard solutions to ensure conformity with the established calibration equations shown in Tables 4.4 and 4.5. The concentration of each was estimated using the appropriate calibration equation. The results are listed below in Tables 4.6 to 4.11.

Table 4.6: Analytical results for the extraction of caffeine from spiked synthetic seawater; Calibration equation Y = 70.8X – 1.3216

Bottle	Mean peak area	Caffeine concentration (mg/L)	%Recovery of caffeine
1	607.9	8.6	83.8
2	515.1	7.29	71.1
3	641.6	9.08	88.5
4	585.3	8.28	80.7
5	665.7	9.42	91.8
Loading solution	725.6	10.26	100
1ppm	68.4	0.99	
10ppm	688.8	9.75	

Table 4.7: Analytical results for the extraction of methylparaben from spiked synthetic seawater; Calibration equation Y = 106.84X - 1.394

Bottle	Mean Peak Area	Methylparaben concentration (mg/L)	%Recovery of Methylparaben
1	982.5	9.21	91.92
2	930.8	8.73	87.13
3	1085.3	10.17	101.5
4	1047	9.81	97.9
5	1066.9	10	99.8
Loading solution	1069.5	10.02	100
1ppm	100.7	0.95	
10ppm	1022.4	9.58	

Table 4.8: Analytical results for the extraction of estradiol from spiked synthetic seawater; Calibration equation Y = 148.6X + 1.4613

Bottle	Mean Peak Area	Estradiol concentration (mg/L)	%Recovery
1	1293.1	8.692	86.8
2	1211.7	8.144	81.3
3	1429.1	9.607	95.9
4	1312.5	8.823	88.1
5	1378.4	9.266	92.5
Loading solution	1489.4	10.013	100
1ppm	154.8	1.032	
10ppm	1587.8	10.675	

Table 4.9: Analytic Results for the extraction of ethynylestradiol from spiked synthetic seawater; Calibration equation Y = 144.04X + 1.241

Bottle	Mean Peak Area	Ethynylestradiol concentration (mg/L)	%Recovery of ethynylestradiol
1	1185.2	8.219	87.3
2	1121.2	7.775	82.6
3	1318.8	9.147	97.1
4	1226.6	8.507	90.3
5	1271.5	8.818	93.6
Loading solution	1357.9	9.418	100
1ppm	150.2	1.035	
10ppm	1541	10.689	

Table 4.10: Analytical results for the extraction of ibuprofen from spiked synthetic seawater; Calibration equation Y = 43.525X + 0.3842

Bottle	Mean Peak Area	Ibuprofen concentration (mg/L)	%Recovery of Ibuprofen
1	16.73	0.38	3.74
2	20.67	0.47	4.63
3	29.53	0.67	6.6
4	9.8	0.22	2.17
5	22.2	0.5	4.93
Loading solution	442.17	10.15	100
1ppm	85.2	1.95	
10ppm	872.8	20.4	

Table 4.11: Analytical results for the extraction of nonylphenol from spiked synthetic seawater; Calibration equation Y = 22.976X – 3.6567

Bottle	Mean Peak Area	Nonylphenol concentration (mg/L)	%Recovery of Nonylphenol
1	144.73	6.46	76.36
2	135.43	6.05	71.51
3	151.27	6.74	79.67
4	148.53	6.62	78.25
5	143.93	6.42	75.89
Loading solution	190.73	8.46	100
1ppm	20.4	1.05	
10ppm	183.87	8.16	

The data listed in Tables 4.6 to 4.11 is summarised in Table 4.12

Table 4.12: Table showing the % recovery of each analyte in each bottle

Bottle	%Recovery Caffeine	%Recovery Methylparaben	%Recovery Estradiol	%Recovery Ethynylestradiol	%Recovery Ibuprofen	%Recovery Nonylphenol
1	83.8	91.9	86.8	87.3	3.7	76.4
2	71.1	87.1	81.3	82.6	4.6	71.5
3	88.5	101.5	95.9	97.1	6.6	79.7
4	80.7	97.9	88.1	90.3	2.2	78.3
5	91.8	99.8	92.5	93.6	4.9	75.9
Mean recovery	83.2	95.6	88.9	90.2	4.4	76.36
STDEV	7.99	5.99	5.58	5.6	1.62	3.11
RSD	9.6	6.27	6.28	6.21	36.82	4.07

Discussion

The results shown in each of the Tables 4.6 – 4.11 show excellent reproducibility for caffeine, methylparaben, estradiol and ethynylestradiol. The % recoveries in each case are typically greater than 80%. Ibuprofen, however, shows poor recovery and less satisfactory reproducibility than the other species studied. Nonylphenol shows recovery in the range 71.5% - 79.7% and good agreement for each bottle measured. These results show the robustness of the method. Typically the RSD (n = 5) is less than 9.6% (with the exception of ibuprofen). This compares with a method for the recovery of estrogens and progestogens from sediment reported by de Alda *et al.* [42] that had an average recovery of 87% with RSD $\leq$11 based on six separate trials.

The method of extraction was considered satisfactory for the analytes, caffeine, methylparaben, ethynylestradiol, estradiol and nonylphenol. The method could not be considered suitable for the recovery of the acidic analyte ibuprofen. However, emphasis of the study was then placed on the species with the highest recoveries.

Chapter 5

Determination of target analytes in marine water samples from Dublin bay

5.1: Introduction

Using the SPE method described in section 3.6 (p. 83) and the analytical methods summarised in Tables 4.2 and 4.3 (p. 94) seawater samples from Dublin bay were extracted and analysed for the presence of the target analytes. This chapter presents the results of the three sampling campaigns described in section 2.5 (p. 66),

(i) Samples taken at the point of discharge of the effluent stream of the Ringsend WWTP into the bay;

(ii) A pilot study;

(iii) The main sampling campaign;

5.2: Point of discharge of the effluent stream

Samples were collected from a position just downstream of the point where the WWTP effluent joins the cooling water exiting from the ESB power station (see plate 2.14, p. 52). Six samples were analysed so that sufficient data would be generated to allow for the identification of any relationship that might exist between the concentrations of the analytes. These samples were extracted using the SPE method described in Section 3.6, (p.83) and the extracts were analysed for the presence of caffeine, estradiol and ethynylestradiol. Analysis of these samples for caffeine was completed on a Hewlett Packard 1100 series instrument. Otherwise the method was as described in Table 4.2 (p. 94). The results of the study are shown in Tables 5.1 and 5.2. Histograms of the mean concentrations of the analytes detected are shown in Figures 5.1 and 5.3.

Table 5.1: Analytical results for the presence of caffeine in effluent samples of volume, 2.77 L. Calibration equation, Y = 67.684X + 8.1467, R^2 = 0.9957. Confidence intervals have been obtained using $t_c \times s/\sqrt{(n-1)}$, where $t_c = t_{95\%, 2\ d.f.}$ for a two tailed test.

	Peak areas from replicate injections	Caffeine concentration in 1 mL extract (mgL^{-1})	Caffeine concentration in effluent (ngL^{-1})	Mean Concentration (ngL^{-1}) (n = 3)	Stdev (ngL^{-1}) (n = 3)	Confidence interval (ngL^{-1})
Sample 1	174.1	2.452	885.2			
	177.1	2.496	901.08			
	178.5	2.517	908.66	898.31	11.97	36.5
Sample 2	150.8	2.108	761.01			
	153.7	2.15	776.17			
	154.9	2.168	782.67	773.28	11.11	33.8
Sample 3	147.9	2.065	745.49			
	143.6	2.001	722.38			
	144.9	2.02	729.24	732.37	11.87	36.5
Sample 4	220.2	3.133	1131.05			
	234.1	3.338	1205.05			
	221.4	3.151	1137.55	1157.88	40.98	124.7
Sample 5	187.1	2.644	954.51			
	186.6	2.637	951.99			
	183.9	2.597	937.55	948.02	9.15	28
Sample 6	133.6	1.854	669.31			
	135.7	1.885	680.51			
	137.2	1.907	688.45	679.42	9.62	29.2

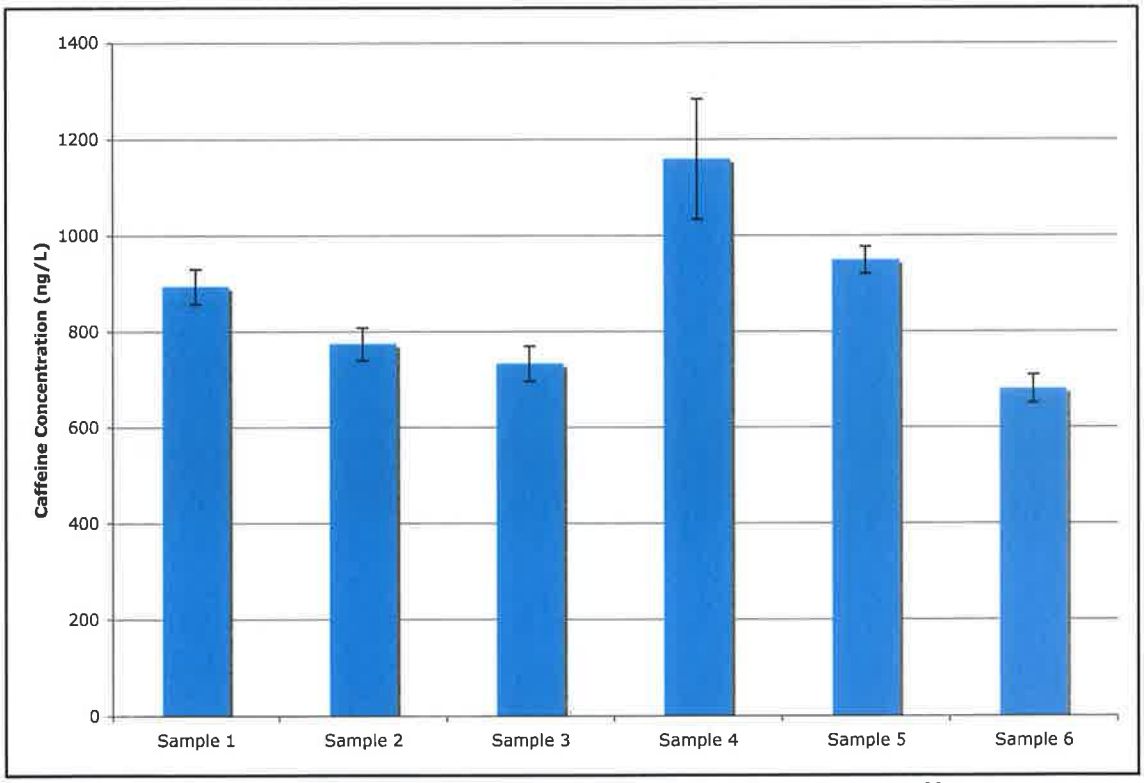


Figure 5.1: Histogram showing the concentration of caffeine in the effluent samples. The error bars represent the confidence intervals as described in Table 5.1.

5.2.1: Comparative concentrations of caffeine in the effluent samples

From this investigation caffeine was detected in each of the six effluent samples at a mean concentration of 864 ngL^{-1}, with a standard deviation of 175.4 ngL^{-1} (n = 6). As the standard deviation shows, there was considerable variation in the concentration of caffeine from one sample to another, even when the confidence intervals are taken into consideration. This may be explained by the turbulence that exists around the point where the WWTP effluent joins the stream of cooling water resulting in an uneven mixing of the two streams at the point of sampling. Another consideration that may be relevant is the existence of several points of exit of the effluent into the cooling water (see plate 2.14, p.52). There may be some difference in composition of the effluent coming from each exit that gives rise to this difference in concentration. Greater knowledge of the WWTP system would be needed before commenting further.

5.2.2: Analysis of effluent samples for estradiol and for ethynylestradiol

Using the methods described above in Table 4.3, (p. 94) the effluent samples were analysed to determine the presence and the concentration of estradiol and ethynylestradiol. A representative chromatogram of a sample is shown in Figure 5.2 superimposed on a chromatogram of a standard solution of the estrogens. From this study estradiol was detected in each of the samples but there was no evidence of ethynylestradiol.

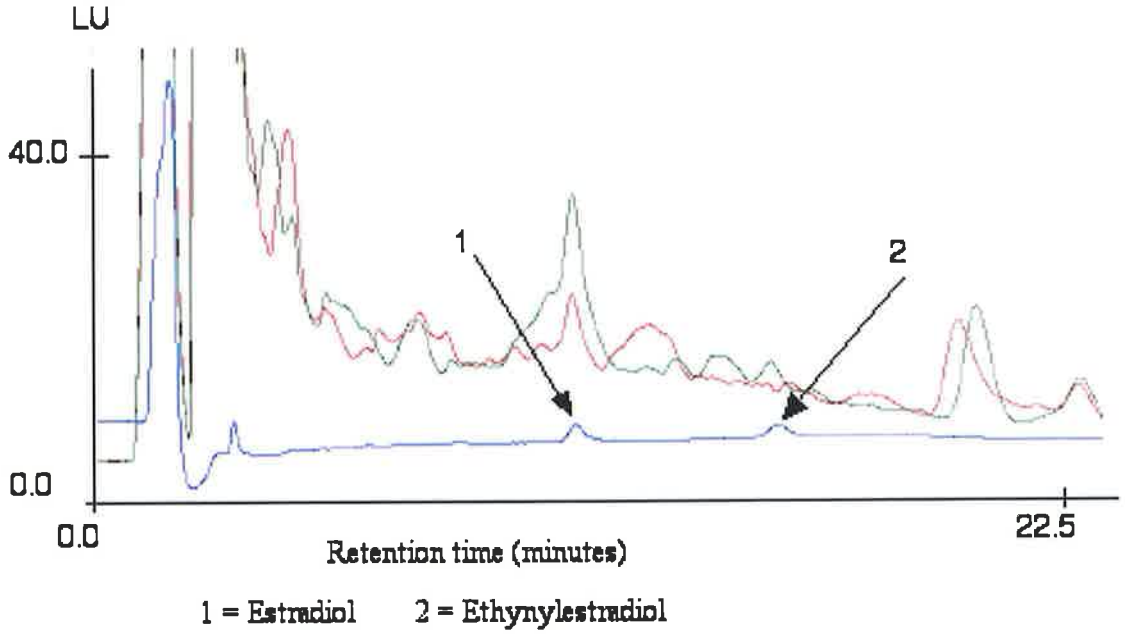


Figure 5.2: Chromatograms of effluent sample 1 (red trace), effluent sample 1 (spiked) (green trace) and a standard solution of estradiol and ethynylestradiol (blue trace). The mobile phase was 45% acetonitrile at 1 mLmin^{-1}, FLD at Ex 230 nm and Em 310 nm.

Table 5.2: Analytical results for presence of estradiol in effluent samples, volume 2.77 L. Calibration equation, Y = 148.6X + 1.4613, R^2 = 1. Confidence intervals have been obtained using $t_c \times s/\sqrt{(n-1)}$, where $t_c = t_{95\%, 2\ d.f.}$ for a two tailed test.

	Peak areas from replicate injections	Concentration estradiol in 1mL extracts	Concentration estradiol in effluent (ngL^{-1})	Mean concentration estradiol in effluent (ngL^{-1}) (n = 3)	Stdev (ngL^{-1}) (n = 3)	Confidence interval (ngL^{-1})
Sample 1	177.8	1.187	428.52			
	188.1	1.256	453.43			
	206.3	1.378	497.47	459.81	34.91	106.16
Sample 2	236.5	1.582	571.12			
	247.9	1.658	598.56			
	268.7	1.798	649.1	606.26	39.56	120.3
Sample 3	140.4	0.935	337.55			
	143.9	0.959	346.21			
	141.4	0.942	340.07	341.28	4.45	13.53
Sample 4	240.4	1.608	580.51			
	243.1	1.626	587			
	245.2	1.64	592.06	586.52	5.79	17.61
Sample 5	185.3	1.237	446.57			
	173.5	1.158	418.05			
	175.3	1.17	422.38	429	15.37	46.74
Sample 6	156.9	1.046	377.62			
	142.4	0.948	342.24			
	158.9	1.059	382.31	367.39	21.91	66.63

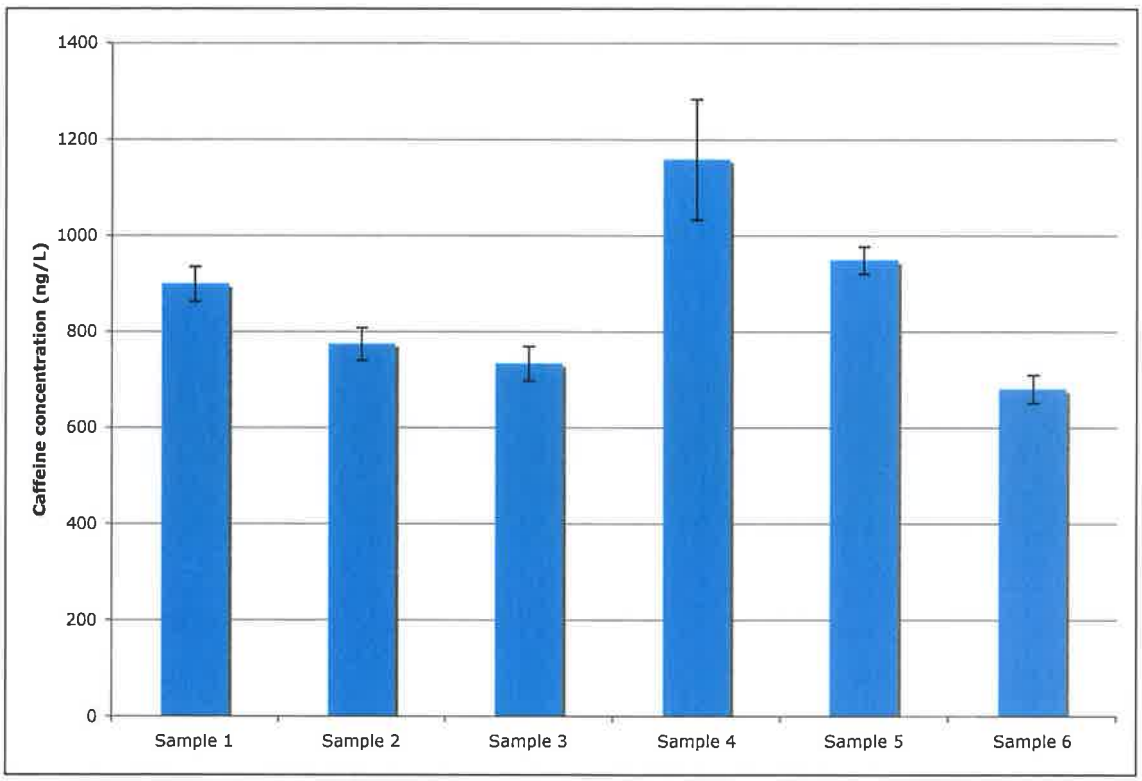


Figure 5.3: Histogram showing the concentration of Estradiol in the WWTP effluent samples. Error bars represent confidence intervals as described in Table 5.2

5.2.3: Comparative concentrations of estradiol in effluent samples

In this investigation estradiol has been detected in each of the effluent samples at a mean concentration of 465 ngL^{-1}, with a standard deviation of 110.3 ngL^{-1} (n = 6). The wide variation in the concentration from one sample to another, that was observed in the case of caffeine, is again apparent in the case of estradiol, though the standard deviation is not as great.

The measured concentration of estradiol in these samples of effluent is considerably in excess of reported values for this compound in comparable matrices, e.g. Liu *et al.* [91] report 14 – 17 ngL^{-1} of this compound in sewage outfall and Braga *et al.* [93] refer to a reported value of 14 ngL^{-1} in treated effluent.

5.2.4: Adjustment of the measured concentrations of caffeine and estradiol in the WWTP effluent samples

To allow for the discrepancy that must exist between the measured and the actual concentration of the analytes in the effluent samples the measured concentrations were adjusted by taking into account the % recovery of the method as determined in Section 4.3, (p. 101). The % recovery of caffeine was 83.2 and of estradiol was 88.9 (see Table 4.12, p. 104). By applying an appropriate multiple to the measured concentration of these two analytes an actual concentration of each was estimated. The results are shown in Table 5.3.

Table 5.3: Measured and adjusted concentrations of caffeine and of estradiol in samples of WWTP effluent

Sample	Measured concentration of Caffeine in effluent (ngL^{-1})	Actual concentration of caffeine in effluent (ngL^{-1})	Measured concentration of estradiol in effluent (ng/L)	Actual concentration of estradiol in effluent (ngL^{-1})
1	893.1	1073.4	459.8	517.2
2	773.3	929.5	606.3	682.0
3	732.4	880.3	341.3	383.9
4	1157.9	1391.7	586.5	659.7
5	948	1139.4	429	482.6
6	679.4	816.6	367.4	413.3

5.2.5: Relative concentrations of caffeine and estradiol in effluent samples

In order to identify any relationship that might exist between the concentrations of the detected analytes the ratio of these concentrations was calculated. Table 5.4 lists the values of the ratios of the concentrations of the two analytes in the effluent samples.

Table 5.4: Concentrations of caffeine and estradiol and their ratios in six samples of WWTP effluent

Actual concentration of caffeine in effluent (ngL^{-1})	Actual concentration of estradiol in effluent (ngL^{-1})	Ratio of concentrations
1073.4	517.21	2.1
929.4	682	1.36
880.3	383.9	2.29
1391.7	659.7	2.1
1139.4	482.6	2.36
816.6	413.3	1.98

The information listed in Table 5.4 is shown graphically in Figure 5.4

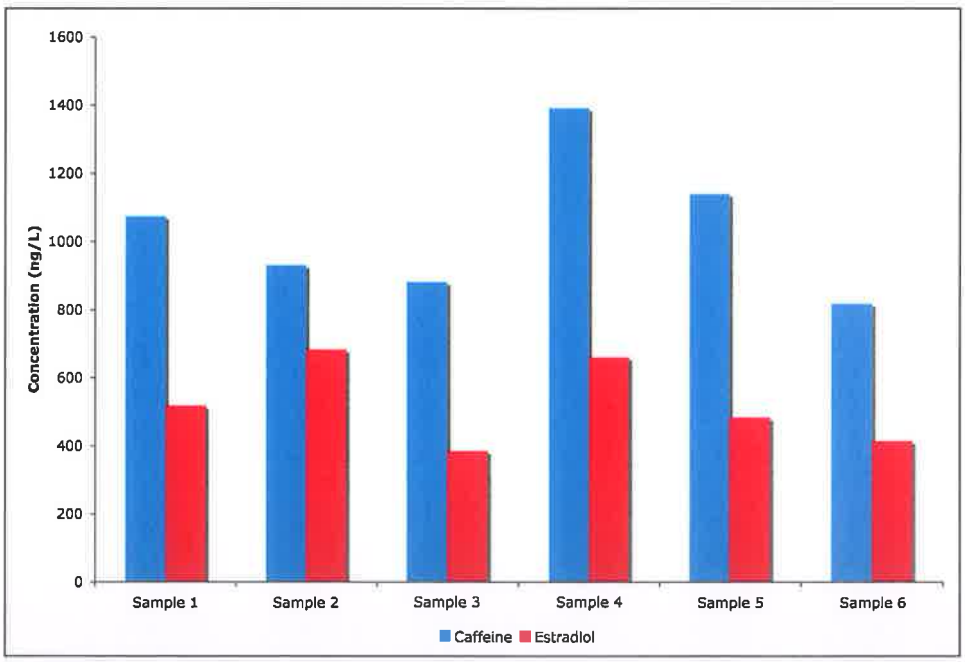


Figure 5.4: Histogram showing the actual concentrations of caffeine and estradiol in effluent samples

Discussion

With the exception of Sample 2 the ratio of the concentrations of the two compounds in the WWTP effluent approaches 2. If this sample is excluded from consideration the mean ratio is 2.16, (RSD 9.3). The consistence of the ratio between the concentrations of caffeine and estradiol supports the view that the reason for the wide variation in concentration from one sample to another is due to turbulence at the point of sampling rather than a difference in composition at different exit points.

5.3: The pilot study

As explained above (Section 2.5.2, p. 66) a pilot study of five of the randomly selected sampling sites was carried out to gain experience in accessing the sampling sites, in collecting the samples and in applying the methods of extraction and analysis to these samples.

The triplicate samples taken from the sites selected for the pilot study were extracted and analysed for caffeine as described above. Table 5.5 lists the results of this analysis and Figure 5.5 shows a histogram of the mean values for each site.

Table 5.5: Analytical results for the presence of caffeine in triplicate samples from five of the randomly selected sampling site

Site	Sample A (ngL^{-1})	Sample B (ngL^{-1})	Sample C (ngL^{-1})	Mean (ngL^{-1})	Stdev (ngL^{-1})	RSD
Site 29	69.6	116.8	90.6	92.3	23.6	25.6
Site 40	94.5	119.7	108.6	107.6	12.6	11.7
Site 70	90.8	82.6	66.2	79.9	12.5	15.6
Site 91	103.8*	20.6	28	24.3	5.23	21.5
Site 107	23.4	24.7	16.9	21.7	4.2	19.4

*Excluded in calculating the mean; see below.

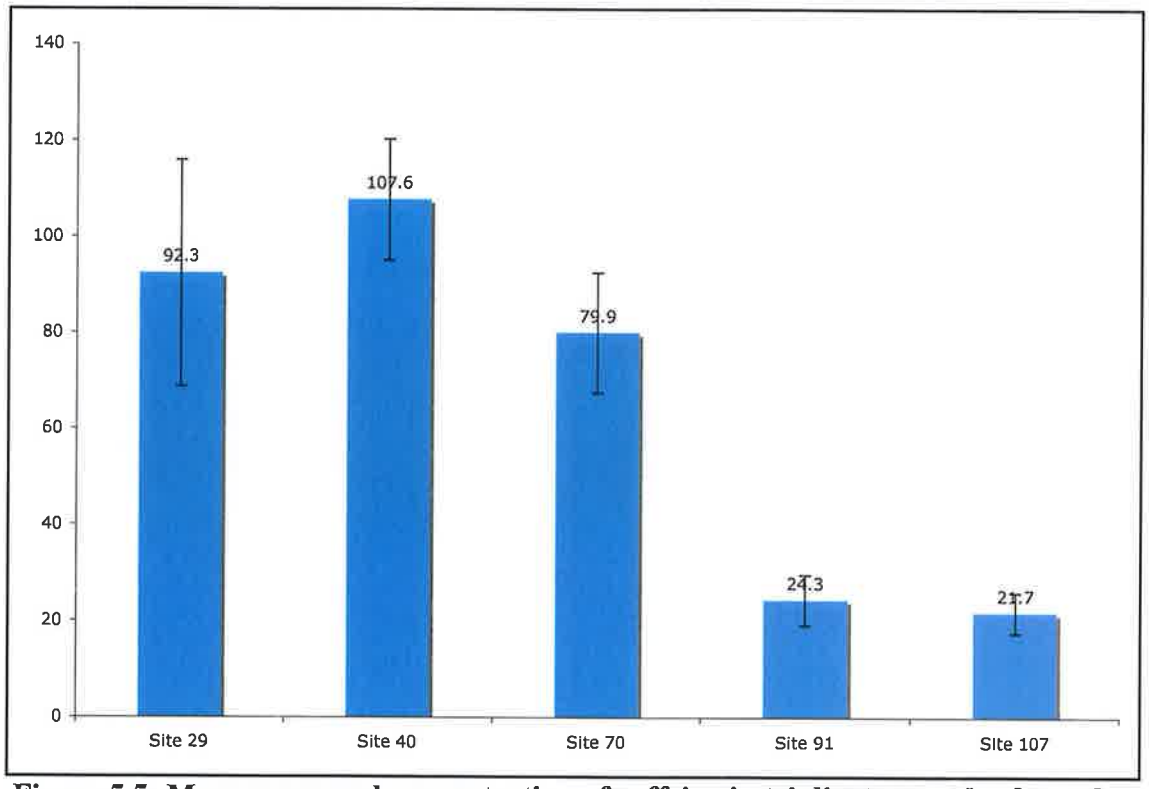


Figure 5.5: Mean measured concentration of caffeine in triplicate samples from the pilot study. Error bars represent the standard deviations within a triplicate set of samples

Discussion

From these investigations caffeine has been detected and quantified in each of the sites chosen for the pilot study, but at considerably lower concentrations than was measured in the effluent samples for which a mean value of 864 ngL^{-1} (n = 6) was obtained. Both Sites 91 and 107 (Plates 2.10 and 2.11) are south of the South Bull Wall. Sites 29, 40 and 70 (Plates 2.3, 2.5 and 2.7) are north of this barrier. The intuitive beliefs that, as a result of dilution and other physical effects, the concentration of anthropogenic analytes would diminish appreciably with distance from the point of exit of the WWTP effluent and that it would be higher north of the South Bull Wall than south of this barrier seem to be supported by these results.

Sites 29 and 40 (see map, Figure 2.1, p. 44) both belong to an area in which free circulation of the water of the bay is impeded by the causeway running from the mainland to the Bull Island. Together with tidal effects this may be a factor in the slightly higher value shown at these sites than at Site 70 which is nearer to the point of discharge of the WWTP effluent and might be expected to show a higher value than was measured.

Site 91 is within the piers of Dun Laoghaire Harbour. There are boats moored within the harbour and several premises both on the piers and along the waterfront that may flush the contents of kitchens sinks etc. into the harbour waters. A localized high concentration of such material may account for the anomalous value achieved for Sample A (Figure 5.5) from this site, rather than experimental error. Nevertheless this value has been excluded from consideration in calculating the mean of the values for each site.

5.4: The main sampling campaign

Completion of the pilot study indicated that samples could be collected from the randomly selected sites and extracted and analysed according to the established potocols. Analysis of the samples collected at the point of discharge of the WWTP effluent had shown the presence of caffeine and estradiol and had suggested the possible existence of a 2:1 ratio in their concentrations.

The main sampling campaign was carried out to estimate the concentration of these analytes in the region represented by the 15 randomly selected sites (Figure 2.1) and to investigate whether any relationship might exist between their concentrations that would allow the concentration of one anthropogenic pollutant to be used as an index for the concentration of others. Thus samples were collected in triplicate from the 15 sampling sites and analysed for caffeine and for estradiol as described above.

5.4.1: Analysis of the extracts for caffeine

After caffeine had been identified and quantified in the extracts one sample from each triplicate set was spiked with a standard solution of caffeine and again analysed. An increase in the area of the relevant peak was accepted as confirmation of the identity of the peak. The results of the analysis of the samples for caffeine are listed in Table 5.6 and shown graphically in Figures 5.6 and 5.7 and in Figure 5.8 the mean measured concentrations of caffeine have been superimposed on the map showing the position of the sampling sites.

Table 5.6: Concentration of caffeine in triplicate samples taken from 15 sampling sites. Each measurement has been calculated from the mean of three peak areas.

	Sample A (ngL^{-1})	Sample B (ngL^{-1})	Sample C (ngL^{-1})	Mean (ngL^{-1})	Stdev (ngL^{-1})	RSD
Site 5	78.88	74.02	64.82	72.6	7.1	9.8
Site 19	422.81	210.33*	433.6	428.2	7.6	15.8
Site 29	106.19	89	94.61	96.6	8.8	9.1
Site 30	35.81	38.32	39.73	38	2	5.3
Site 40	61.09	77.11	71.34	69.8	8.1	11.6
Site 50	71.34	71.65	65.73	69.6	3.3	4.7
Site 70	283.62	296.72	310.28	296.9	13.3	4.5
Site 72	92.4	93.78	94.09	93.4	0.9	1
Site 74	30.39	31	33	31.5	1.4	4.4
Site 76	36.82	39.13	33.45	36.5	2.9	7.9
Site 82	25.89	38.9	28.54	31.1	6.9	22.2
Site 91	36.6	44.79	58.09	46.5	10.8	23.2
Site 107	29.94	27.41	38.22	31.9	5.7	17.9
Site 110	32.26	26.23	33.1	30.5	3.7	12.1
Site 112	32.82	30.38	33.5	32.2	1.6	5

*This anomalous result has been excluded from the calculation of the mean for this site. See below.

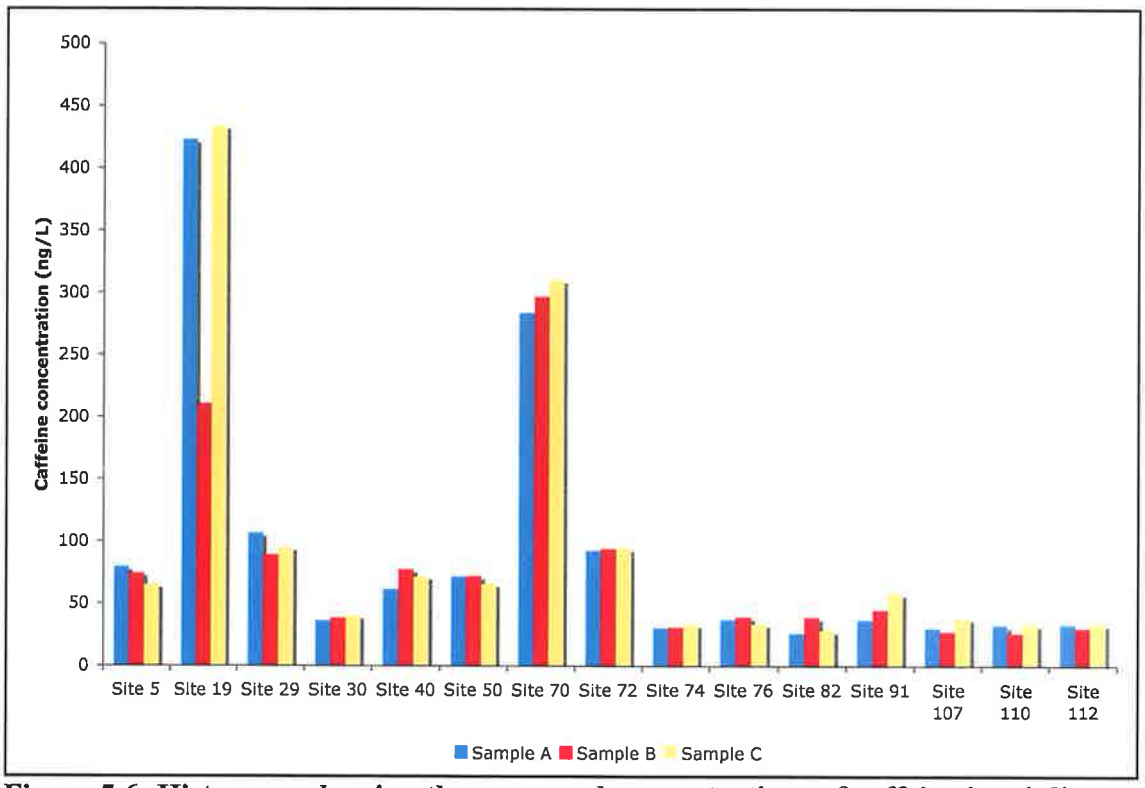


Figure 5.6: Histogram showing the measured concentrations of caffeine in triplicate samples taken from fifteen randomly selected sampling sites

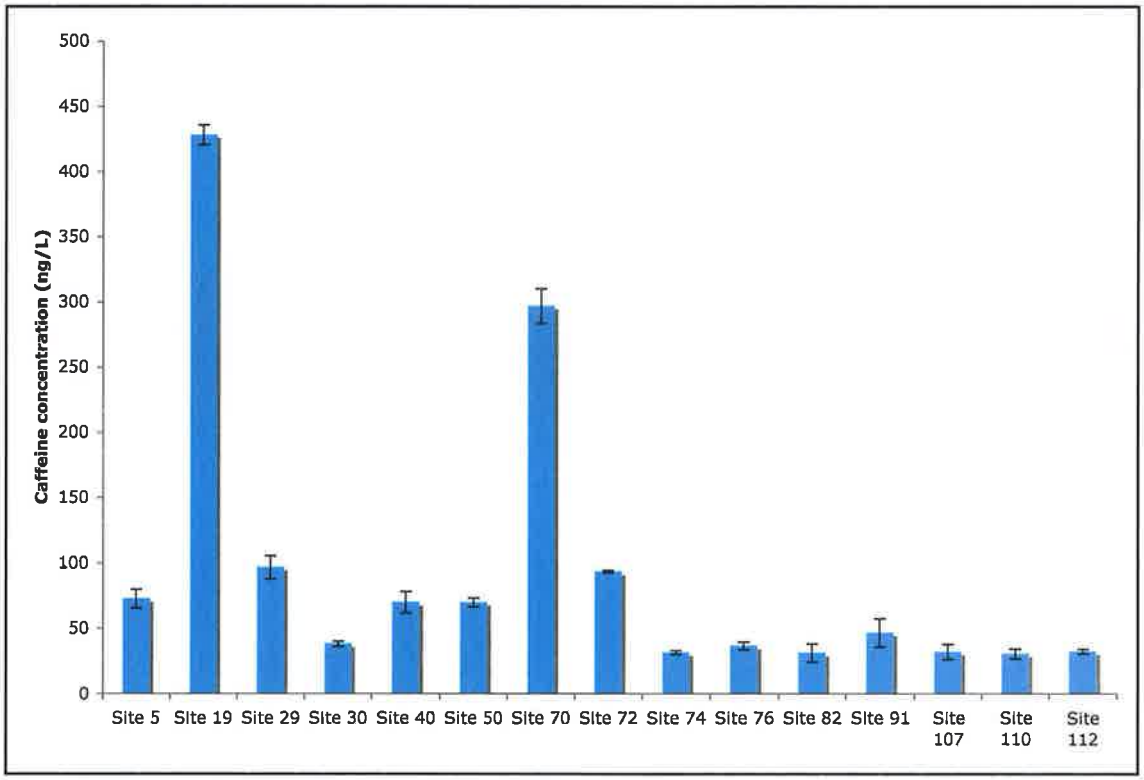


Figure 5.7: Histogram showing mean measured concentration of caffeine in fifteen randomly selected sampling sites (error bars represent the standard deviations of the concentration of the three samples taken from each sampling site). The anomalous value measured for Sample B, Site 19 (see Fig. 5.6), has not been included in the calculation of the mean for this site.

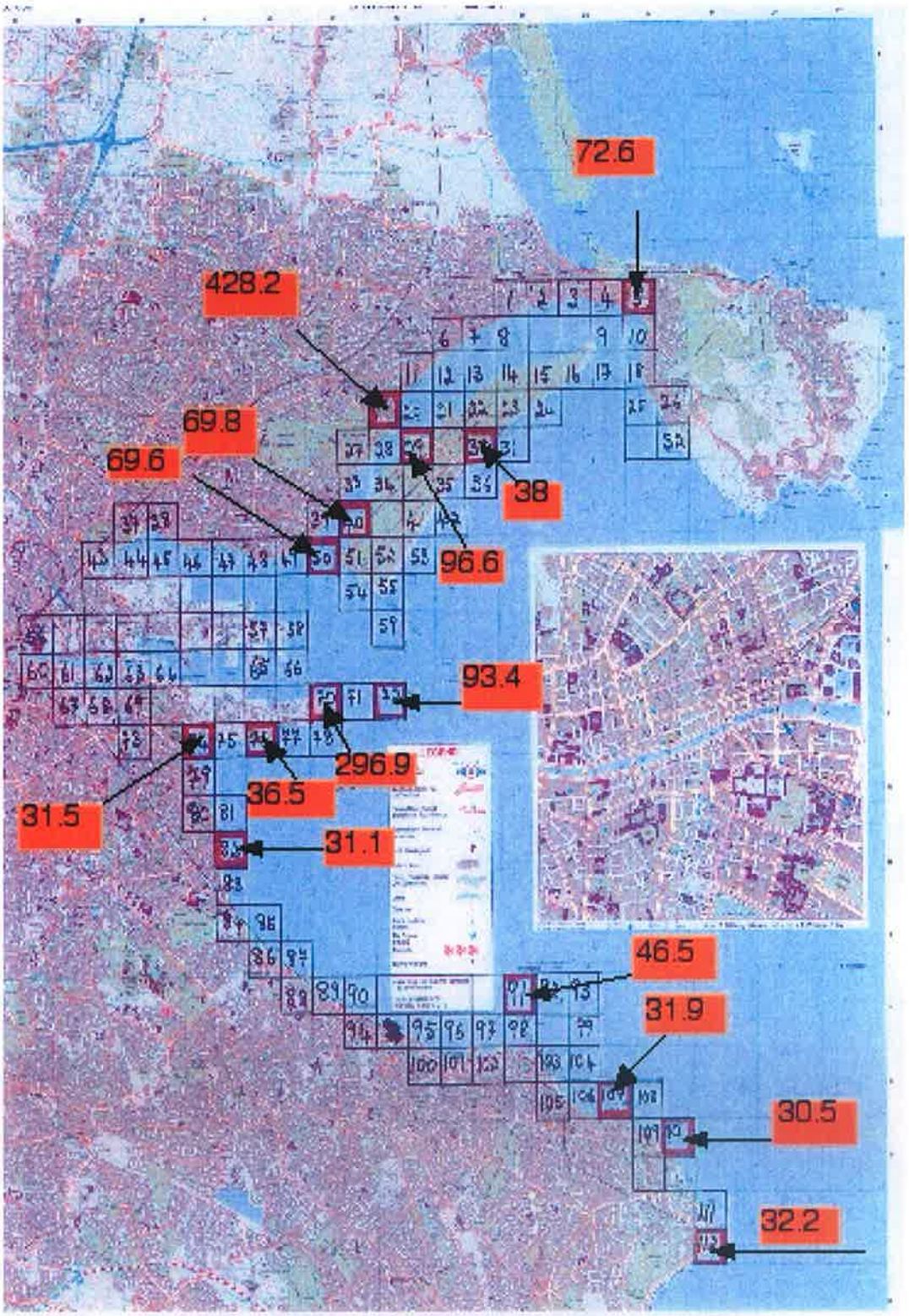


Figure 5.8: Reproduction of map shown in Figure 2.1 with measured concentrations of caffeine shown (concentrations in ngL^{-1})

5.4.2: Distribution of caffeine within the bay

The pattern in the distribution of caffeine in the seawater of the bay that was detected with the limited number of samples analysed in the pilot study has been repeated with the greater number of samples taken in this more extensive sampling campaign. From the results of this investigation it was found that, with the exception of the samples collected from site 19, the concentration of caffeine in the seawater samples was found to decrease as distance from the point of discharge of the WWTP effluent increased. All the samples, with one exception, taken from north of the South Bull Wall had a concentration of caffeine greater than in those taken from sites south of this barrier. The unexpectedly high value obtained at site 19 can, possibly, be explained by the presence of a sizeable stream that flows through the suburbs of Santry, Coolock and Raheny and opens into the bay at this site. This stream may collect the overflow from domestic drains, etc. resulting in a higher than expected reading for the samples from this site. The anomalous result achieved for sample B at this site (see Figure 5.6) may be explained by the uneven distribution of the riverwater on its immediate entry into the seawater of the bay.

The slightly higher value measured at site 91 (see Figure 5.7) than was measured in the surrounding waters may be explained by the considerations discussed in Section 5.3.

Site 70 is along the South Bull Wall, about 500 m from the point of discharge of the WWTP. The value measured for the concentration of caffeine at this site is about 30% of the mean value measured at the point of discharge.

Sites 29, 40 and 50 are all bound to the south by the South Bull Wall and to the north by the causeway from the mainland to the Bull Island. The sediment in this area is almost completely exposed at low tide. The incoming tide, flowing past the point of discharge of the effluent, must fill this area with seawater that contains an appreciable concentration of the constituents of that discharge.

The value measured at site 30 is slightly lower than other values north of the South Bull Wall. This discrepancy, possibly, reflects the more exposed position

of this site. It is outside of the North Bull Wall, though still protected from the open sea by Howth Head.

5.4.3: Chromatograms

Chromatograms of representative samples are shown in Figures 5.9(a) and 5.9(b) superimposed on chromatograms of the same samples after spiking with a standard solution of caffeine. Chromatograms of other samples are shown in Appendix 1.

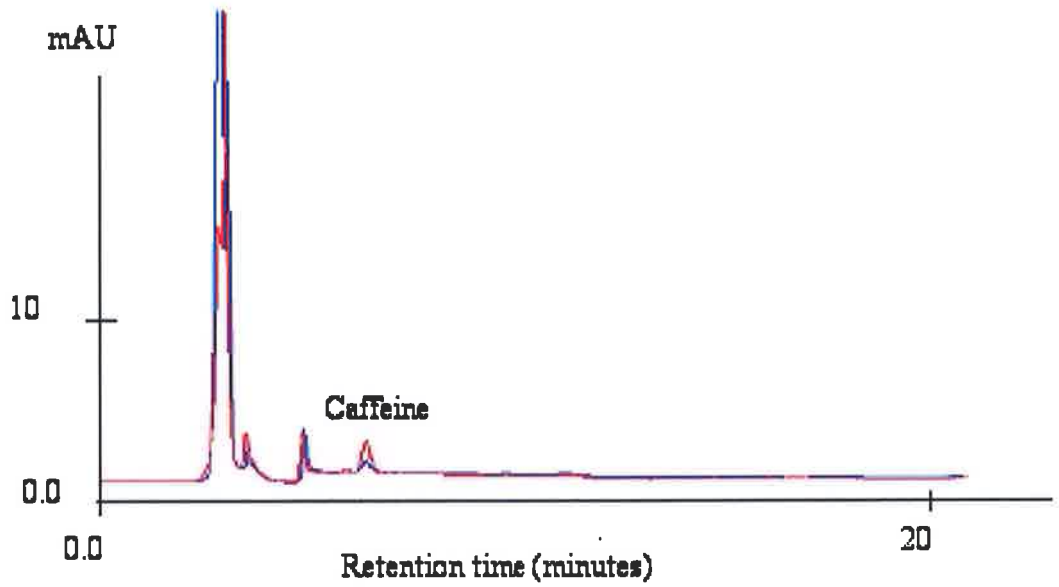


Figure 5.9(a): Chromatograms of Sample 50A before (blue trace) and after (red trace) spiking. Retention time of caffeine 6 minutes with 20% acetonitrile at 0.8 mL min^{-1}. Detection at 272 nm

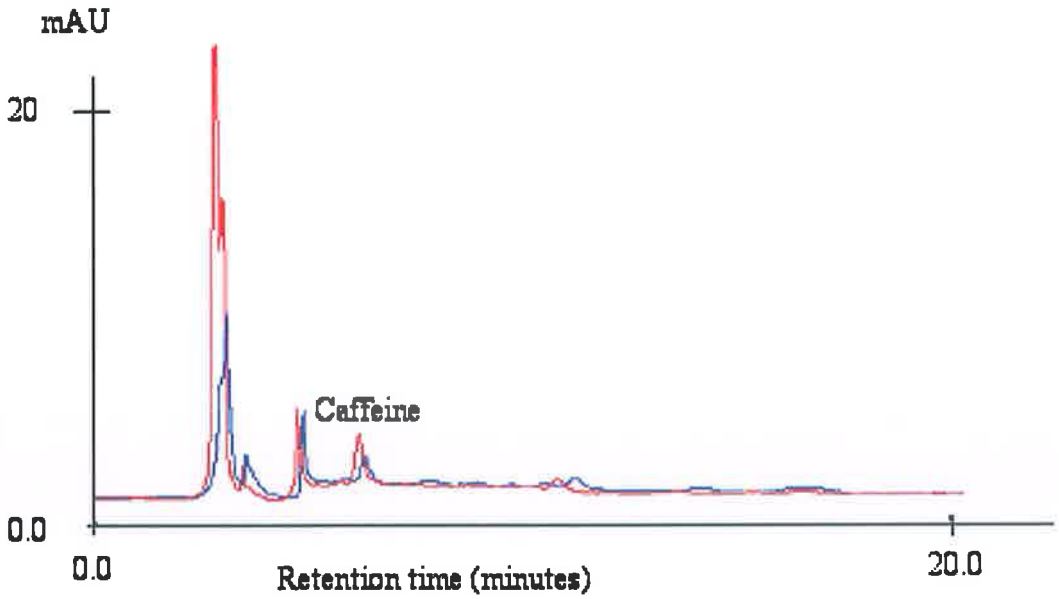


Figure 5.9(b): Chromatograms of Sample 72A before (blue trace) and after (red trace) spiking. Retention time of caffeine 6 minutes with 20% acetonitrile at 0.8 mL min^{-1}. Detection at 272 nm.

5.4.4: Analysis of the extracts for estradiol:

After analysis of the extracts for caffeine had been completed the same extracts were analysed for the presence of estradiol using the established method for the identification and quantification of this compound summarized as in Table 4.3. After the presence of this compound had been identified and quantified in the extracts one sample in each triplicate set was spiked with a standard solution of this compound and analysed once again. An increase in the area of the relevant peak was accepted as confirmation of the identity of that peak.

Table 5.6: Analytical results for the presence of estradiol in triplicate samples taken from fifteen randomly selected sampling sites.

	Sample A (ngL^{-1})	Sample B (ngL^{-1})	Sample C (ngL^{-1})	Mean Concentration (ngL^{-1})	Stdev (ngL^{-1})	RSD
Site 5	296.3	357.8	275.9	310	42.63	13.8
Site 19	265.7	228.8	248.7	247.73	18.47	7.5
Site 29	311.3	221.6	204.2	245.7	57.47	23.4
Site 30	298.3	221.3	392.6	304.07	85.8	28.2
Site 40	230.9	332.4	204.2	255.83	67.64	26.4
Site 50	131.6	125.2	157.6	138.13	17.16	12.4
Site 70	271.5	248.6	268.3	262.8	12.4	4.7
Site 72	350.1	223.8	267.1	280.33	64.18	22.9
Site 74	194.6	219.2	229.9	214.57	18.1	8.4
Site 76	269	152.4	261.2	227.53	65.18	28.6
Site 82	285.5	178	200.6	221.37	56.68	25.6
Site 91	56.3	44.6	29.3	43.4	13.54	31.2
Site 107	179.5	196	199.7	191.73	10.75	5.6
Site 110	–	–	48.9	48.9	–	–
Site 112	52.7	88.3	180.3	107.1	65.84	61.5

The data that is listed in Table 5.6 is shown graphically in Figures 5.10 and 5.11.

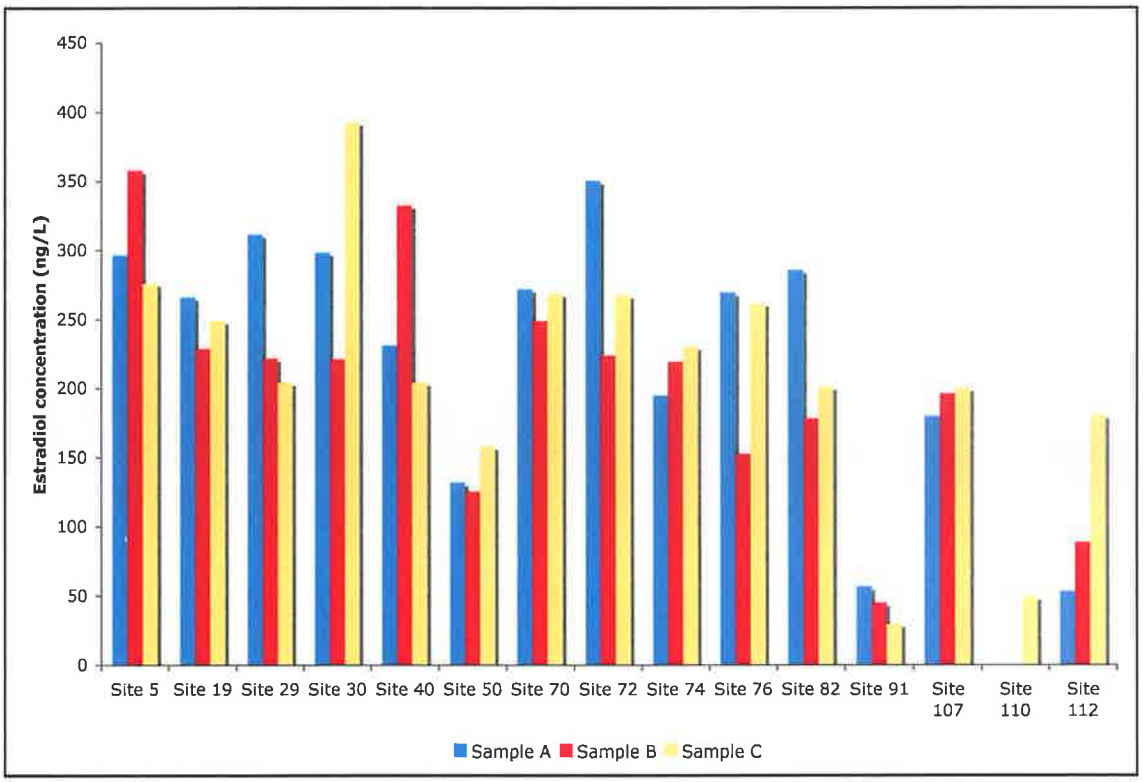


Figure 5.10: Histogram showing the measured concentrations of estradiol in triplicate samples taken from fifteen randomly selected sampling sites.

As can be seen from Figure 5.10 there is considerable variation in the concentration of estradiol in the three samples taken from within many of the sampling sites. Such a large variation was not observed in the concentration of the caffeine in the triplicate samples (see Figure 5.6). Standard deviations are represented as error bars in the histogram of the mean concentrations at each sampling site shown in Figure 5.11.

This large variation in the concentration of estradiol in the samples taken from many of the sampling sites may be a consequence of the lapse of time between collecting and extracting the samples and analyzing them for the steroids. During this time the samples were analysed for caffeine. Despite being kept in airtight sample tubes some evaporation of solvent may have occurred resulting in an increased, but variable, concentration of the analytes.

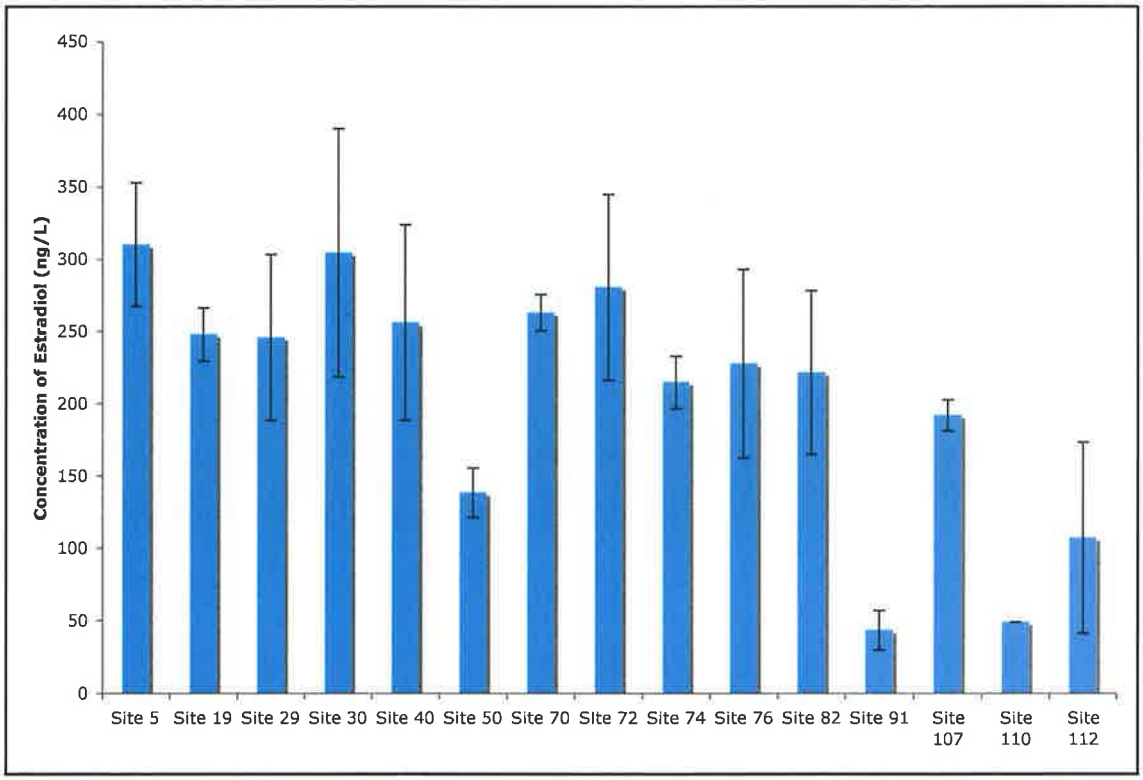


Figure 5.11: Mean measured concentration of estradiol at each of the randomly selected sampling sites. Error bars represent the standard deviations of the three samples contributing to each mean value.

5.4.5: Distribution of estradiol within the bay

From these investigations it was found that estradiol was present at each of the randomly selected sampling sites within the bay at concentrations less than the mean value measured for the effluent samples (465 ngL^{-1}). The decrease in the concentration from this mean value was not as great as the corresponding decrease that was observed for caffeine.

The pattern that was observed for the distribution of caffeine within the bay, i.e. greater concentration on the north side of the South Bull Wall than on the south side, is repeated for estradiol, but, the contrast is not so pronounced, e.g. the mean values of the concentration of estradiol in the samples taken from the south side, i.e. sites 74 to 112, is 151 ngL^{-1} compared with a mean value of 255 ngL^{-1} for those samples taken from north of this barrier. The median value of the former set of samples is 192 ngL^{-1} and of the latter is 259 ngL^{-1}.

The hydrophobic nature of the steroid may cause quantities of this compound to adhere to the sediment of the bay resulting in an equilibrium value of the concentration within the aqueous phase to be, largely, unaffected by the constant accretions from the WWTP effluent.

5.4.6: Chromatograms

Chromatograms of the samples and of the samples after spiking are shown below Figures 5.12(a) – (b). In each case the blue trace is that of the sample and the red trace is that of the same sample after spiking.

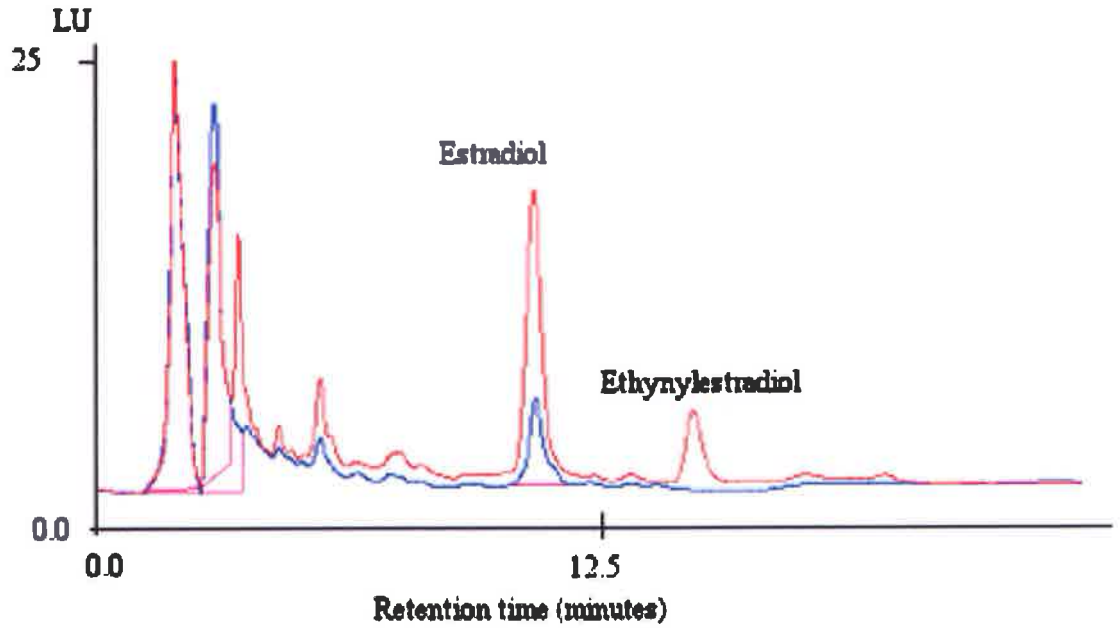


Figure 5.12(a): Chromatograms of Sample 5A, before (blue trace) and after (red trace) spiking. Retention time of estradiol 11.8 min with 45% acetonitrile at 1 mL min^{-1}. FLD Ex 230 nm, Em 310 nm.

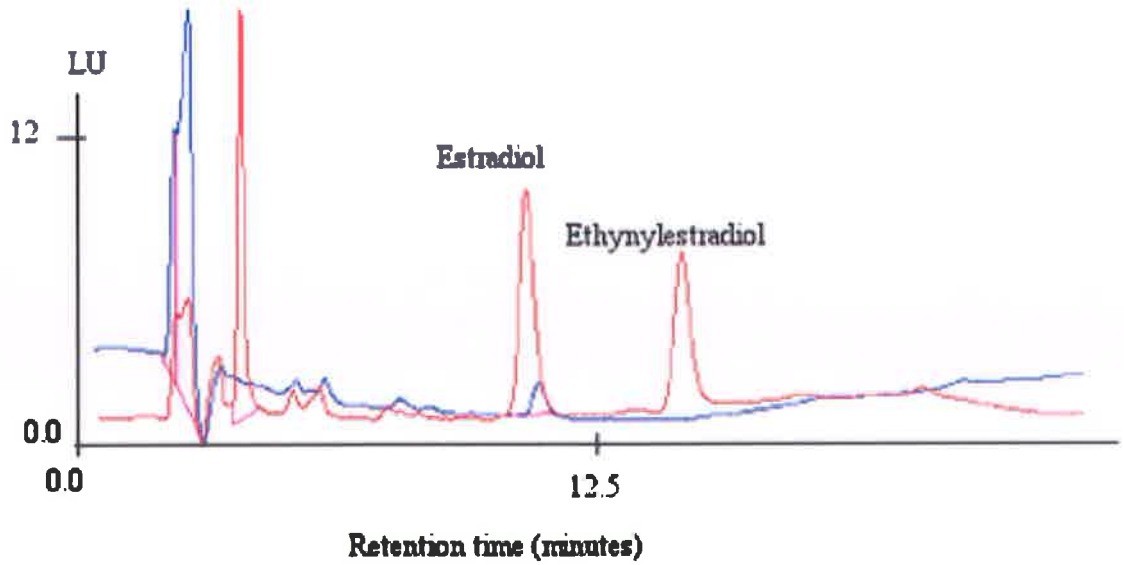


Figure 5.12(b): Chromatograms of Sample 91A, before (blue trace) and after (red trace) spiking. Retention time of estradiol 11.8 min with 45% acetonitrile at 1 mL min^{-1}. FLD Ex 230 nm, Em 310 nm

5.5: Relative concentrations of caffeine and estradiol

The ratio of 2:1 that was observed between the concentrations of caffeine and of estradiol in the effluent samples was not observed within the water of Dublin Bay, in fact, the mean concentration of the steroid within a sampling site, in all but two, cases exceeded that of caffeine. A large reservoir of the more hydrophobic compound in the sediment of the bay might be a factor in reversing the ratio of these two compounds from that observed in the WWTP effluent.

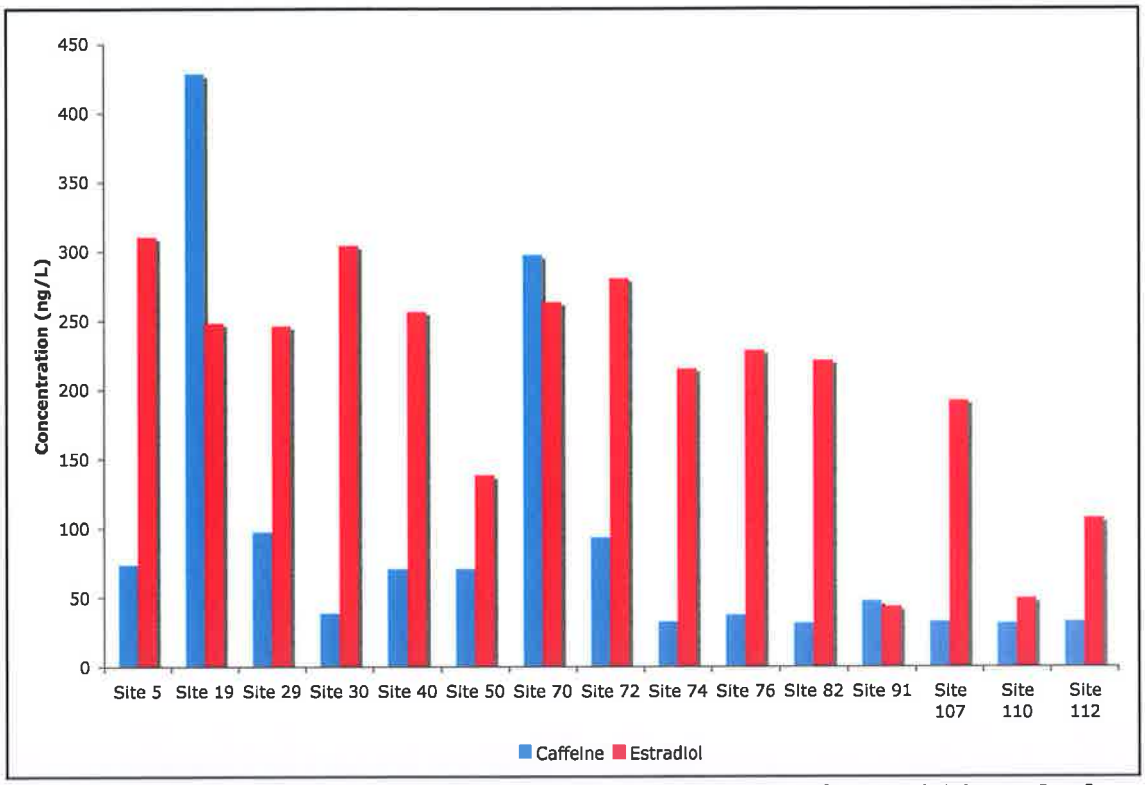


Figure 5.13: Mean measured concentrations of caffeine and of estradiol in each of the fifteen randomly selected sampling sites

5.5.1: Adjustment of the measured analyte concentrations

The measured concentrations of the analytes caffeine and estradiol were adjusted using the established %recovery as determined in Section 4.3, (p. 101) of the SPE method (Table 3.8, p. 83) as estimated by the established HPLC techniques (Tables 4.2 and 4.3, p. 94). This was done to allow for the inevitable discrepancy between the actual concentration of these analytes in the seawater and their concentration as measured by these methods. The recovery rates of these compounds have been selected from Table 4.12, (p. 104) and are reproduced in Table 5.7. The actual concentration of these analytes was estimated by taking an appropriate multiple of the measured concentration. The results of the calculation are shown in Table 5.8

Table 5.7: %Recovery of Analytes (Selected from Table 4.12)

Analyte	%Recovery
Caffeine	83.2
Estradiol	88.9

Table 5.8: Measured and adjusted concentration of caffeine and estradiol in seawater samples taken from 15 randomly selected sampling sites

	Measured concentration of caffeine (ngL^{-1})	Adjusted concentration of caffeine (ngL^{-1})	Measured concentration of estradiol (ngL^{-1})	Adjusted concentration of estradiol (ngL^{-1})
Site 5	72.6	87	310	349
Site 19	428.21	515	247.73	279
Site 29	96.6	116	245.7	276
Site 30	38	46	304.07	342
Site 40	69.8	84	255.83	288
Site 50	69.6	84	138.13	155
Site 70	296.9	357	262.8	296
Site 72	93.4	112	280.33	315
Site 74	31.5	38	214.57	241
Site 76	36.5	44	227.53	256
Site 82	31.1	37	221.37	249
Site 91	46.5	56	43.4	49
Site 107	31.9	38	191.73	216
Site 110	30.5	37	48.9	55
Site 112	32.2	39	107.1	120

5.6: Comparison of results with relevant reported results

For the purposes of comparison Tables 2.2 and 2.6 are reproduced below.

(Table 2.2: Reported values for the concentrations of caffeine in environmental waters.)

Matrix (Country)	Concentration	Reference
River water (Holland)	500 ngL^{-1}	13
Sewage Influent (Norway)	104 μgL^{-1}	18
Sewage effluent (Norway)	0.07 μgL^{-1}	18
Seawater (North sea)	(2.0-16.1) ngL^{-1}	19
Surface Water (USA)	(0.003-1.44) μgL^{-1}	85
Estuary (Miami)	41.2 ngL^{-1}	24
Seawater (Biscayne Bay)	11.9 ngL^{-1}	24
Boston Harbour	(140 -1600) ngL^{-1}	63

(Table 2.6: Reported values for the occurrence of estradiol in environmental matrices)

Matrix	Reported values	Reference
Ocean sediment	0.9ngg^{-1}	93
Treated effluent	14ngL^{-1}	93
Sewage outfall	14 – 17ngL^{-1}	91
STP effluent	< 0.8ngL^{-1}	41
Seawater (Baltic)	< 0.3ngL^{-1}	40
River Water	0.6 – 1.2ngL^{-1*}	25

* This figure represents a combined value for estrone and estradiol

Discussion

The concentrations of caffeine that have been measured in this project are, largely, in accordance with the values that have been reported for comparable matrices elsewhere. However, as Table 2.6 (above) shows, the measured concentrations of estradiol are grossly in excess of reported values. This discrepancy cannot but be accepted as an argument for the exercise of caution in interpreting the significance of the results of this project for the concentrations of estradiol in the waters of Dublin bay.

Chapter 6

Conclusion

6.1: Introduction

As described in section 1.4 (p. 39) the aim of the project was to identify a single anthropogenic pollutant that could be used as an index of more widespread pollution. In pursuit of this aim it was necessary to choose a set of compounds deemed to have the potential for anthropogenic pollution, to select a site within which the target compounds would be likely to occur and to develop methods for their extraction and quantification. Dublin bay was chosen as the sampling area. Six compounds were targeted to be extracted by solid phase techniques and detection and quantification was by HPLC with either UV or fluorescence detection.

6.1.1: The sampling site

The Ringsend WWTP collects and treats wastewater from an area populated by over 1×10^6 individuals and delivers the effluent into the water of Dublin bay. Numerous reports in the literature indicate that such a matrix is likely to contain a range of pollutants that would include steroids, substituted phenols and pharmaceutical residues. There are numerous positions around the bay where samples can be easily collected. For these reasons Dublin bay is considered to be ideal for the overall aim of the project.
In this project the sampling sites were chosen by random selection. This ensured that their selection was unbiased. Fortuitously, the set of sampling sites was also considered to be representative of the bay. Perhaps an equally representative, and no less biased, selection of sites could be made by the careful selection of particular sites as described by Roberts and Thomas [83] in their analysis of the water in the Tyne estuary.

6.1.2: Analytical methods

HPLC methods for the identification and quantification of the analytes caffeine, methylparaben, estradiol, ethynylestradiol, ibuprofen and nonylphenol were developed. These methods were linear and had satisfactory LODs and LOQs. Success in the development of a single isocratic method for the analysis of these compounds that was

practicable was not achieved. Though the separate methods that were developed were effective the length of time between the analyses of the seawater samples using these separate methods allowed for deterioration in the quality of the samples. Since the seawater samples were initially analysed for caffeine and somewhat later for estradiol it is believed that the former set of results was more indicative of the concentration of this analyte in the seawater of Dublin bay than was the latter set indicative of the concentration of estradiol.

6.1.3: SPE method

The mean recovery rate of the SPE (section 4.3, p. 101) method was estimated to be > 76% for each of the analytes except for ibuprofen. The lower pK_a value of this compound ensured that it would be present in anionic form in the neutral, or slightly alkaline, seawater matrices hence the failure of a method which utilised a predominantly reversed phase sorbent, Strata-X, was not surprising.

The established method did not include acidification of the seawater sample since this procedure favoured the co-extraction of material which, when the resulting sample was analysed, gave a chromatogram with a broad undifferentiated peak that obscured the signal for caffeine and those analytes with short retention times.

The difficulty of extracting and quantifying ibuprofen simultaneously with the other target analytes was not resolved in this project. An approach that might have been attempted was that reported by Verenitch *et al.* [76]. In this study a 2 L sample was divided into two 1 L aliquots one of which was adjusted to pH 2 and the other to pH 7.5. Acidic drugs were extracted from and quantified in the former and caffeine was extracted from and quantified in the latter.

The items of glassware that were designed for the project (section 3.7, p. 84) proved to be both cost effective and most satisfactory in facilitating the simultaneous preparation of up to 12 samples suitable for HPLC analysis.

6.1.4: Recovery rate of the method

The standard deviations of the % recovery rate of the different analytes (see Table 4.12, p. 104) were sufficiently large to have suggested that one or other of the factors that influenced this recovery rate might have been controlled in some way that would have given higher mean recovery rates and lower values for the standard deviations. It is suggested that a factor that had potential for uncontrolled variation was the flow rate of the seawater through the sorbent. The Luer stopcocks (see Figure 3.6, p. 84) were difficult to manage in such a way that a constant flow over a 6 hour period was maintained. It is suggested that in any future work attention to this factor might give a higher mean recovery rate.

6.1.5: Concentrations of the analytes in seawater

As stated above the concentrations of caffeine in the seawater of Dublin bay, as determined by this project, were, largely, comparable to the reported concentrations of this compound in similar matrices in other countries. The manner in which the concentration values were distributed within the bay was in accordance with expectation. High concentrations were found in the vicinity of the discharge of the effluent from the Ringsend WWTP and these values decreased with increasing distance from this point.

It was found that the South Bull Wall, which forms the southern rampart of Dublin port, formed a dividing line between regions of high caffeine concentration and of somewhat lower concentration. The higher concentrations were found on the northern side of this wall which is the position where the WWTP effluent is discharged.

Though, for the reasons stated above, the values found for the concentration of estradiol are not considered to be as reliable as those for caffeine, these values did follow the pattern shown by caffeine. There were high concentrations of estradiol in the vicinity of the discharge from the WWTP and these values decreased with increasing distance from this position. Also higher concentrations were found on the northern side of the South Bull Wall than on the southern side of this barrier.

6.1.6: Ratio of the concentrations of caffeine and of estradiol

The ratio of the concentrations of caffeine and of estradiol in the seawater samples taken at the point of discharge of the WWTP effluent seemed to approximate to 2:1. This ratio was not maintained in the samples collected throughout the bay. Further research might establish the reliability of this suggested ratio and whether it is maintained in the effluent over a lengthy period of time.

6.2: Suggestions for further research:

Of the six target compounds that were chosen there was ample evidence in the literature to suggest that caffeine, estradiol, ethynylestradiol, ibuprofen and nonylphenol might be found in the selected sampling region. The widespread use of methylparaben (see section 2.2.2.2, p. 55) was considered to be a substantial reason for believing that its presence might also be detected, especially in the seawater close to the exit of the WWTP effluent.

The project did not achieve success in identifying the analytes methylparaben, ethynylestradiol, ibuprofen or nonylphenol in the seawater samples, even in those samples collected at the point of discharge of the WWTP effluent. In view of the numerous reports in the literature that such compounds, with the exception of methylparaben, have been found in comparable matrices elsewhere it is believed that, with modifications of the procedures described in this project, it should be possible to find evidence of such compounds in the seawater of Dublin bay.

The overall aim of the project, i.e. the identification of one pollutant that might act as an index for others, awaits further research that might identify the concentration of a range of anthropogenic pollutants in a variety of different locations.

Despite the caution that has been suggested regarding the values for the concentration of estradiol in the seawater found by this project the prevalence of this compound in the estuary of the river Liffey does seem to warrant further research. The possibility that there might be a large amount of this hydrophobic compound adsorbed to the sediment of the bay giving an equilibrium value in the aqueous phase might be investigated. The possibility of a causal connection between an apparently high concentration of estrogenic

material in the seawater of the bay, and hence in the estuary of the river, and the decline in the salmon stock of the river might also be investigated.

According to reports in the literature, e.g. Arsenault *et al.* [39], low level exposure of young salmon in the parr-smolt transformation stage has a detrimental effect on their ability to survive in the open sea. Since this transformation stage occurs as the fish are returning to the sea from the river in which they were spawned the number of fish that will survive and eventually return to this river is compromised.

Like many Irish rivers salmon stocks in the river Liffey have declined in recent years. Presumably there are many reasons for this decline. In view of the reported effects of high concentration of estrogenic material on young salmon the concentration of estradiol in Dublin bay established by this project might be considered to be a possible contributing factor.

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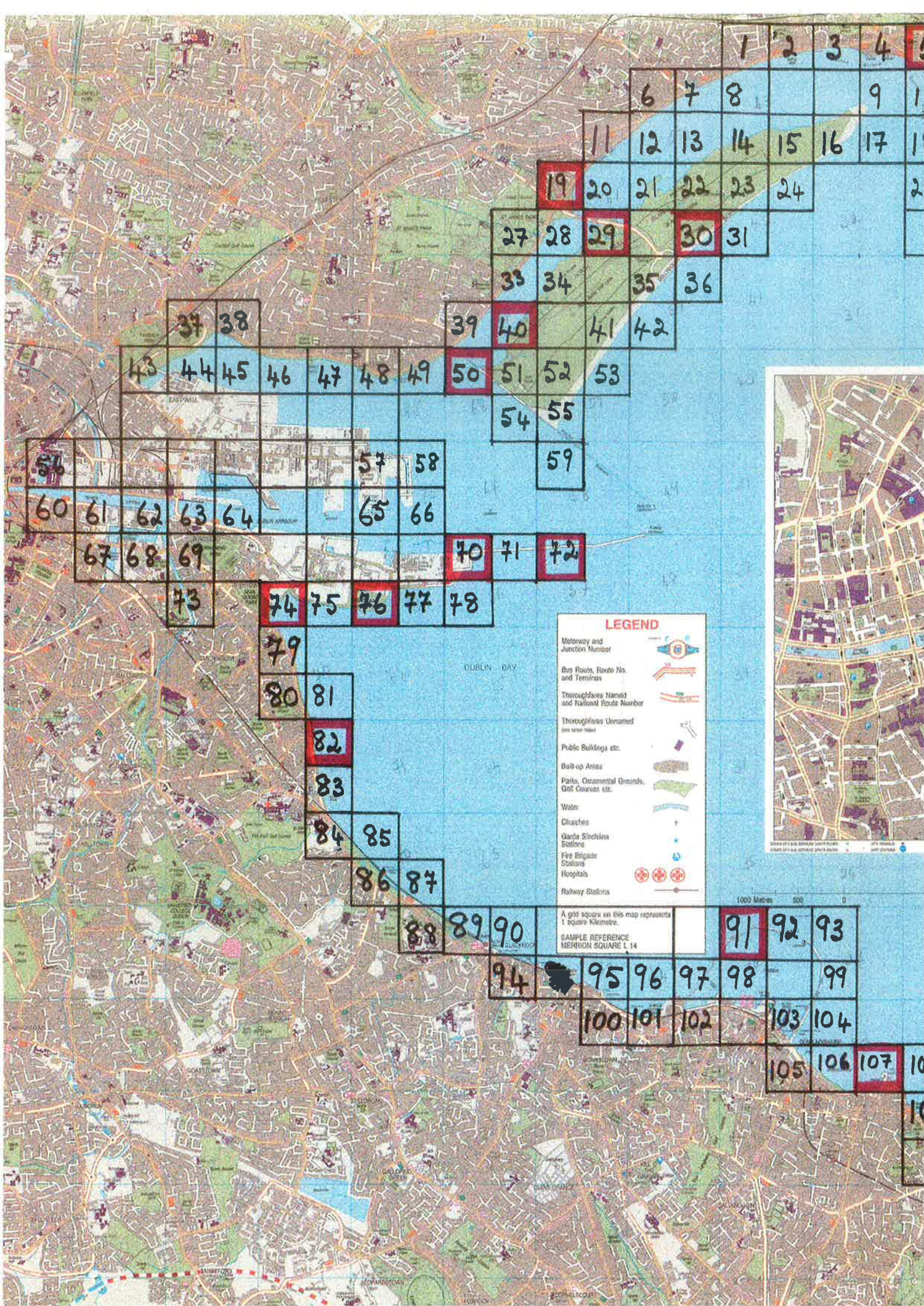
LEGEND
Motorway and Junction Number
Bus Route, Route No. and Terminus
Thoroughfares Named and National Route Number
Thoroughfares Unnamed
Public Buildings etc.
Built-up Areas
Parks, Ornamental Grounds, Golf Courses etc.
Water
Churches
Garda Síochána Stations
Fire Brigade Stations
Hospitals
Railway Stations
A grid square on this map represents 1 square Kilometre.
SAMPLE REFERENCE
MERRION SQUARE L 14
DUBLIN BAY
1000 Metres 500 0