I - Executive Summary

1. INTRODUCTION

Changing land use patterns, global climate change, and expanding human populations along coast lines, including those of the Mediterranean and Black Seas, are putting increased stress on coastal regions and may have serious impacts on public health. Sewage, both treated and raw, which enters into receiving waters may carry diverse inorganic and organic contaminants as well as pathogenic microorganisms, all of which may affect both marine populations as well as their human consumers. While a number of such contaminants (*e.g., chlorinated hydrocarbons, methylmercury, polycyclic aromatic hydrocarbons*) are relatively well documented in their effects on marine and human populations, other more novel contaminants are starting to be recognized as having worrisome effects. These may include a new generation of insecticides, herbicides (*e.g. carbamate and organophosphate pesticides)*, and other novel organic compounds used in industry *(e.g. food additives)*, pharmaceutically active compounds which may influence hormonal function in marine animals and in human consumers, antibiotics which may enter coastal communities through sewage and mariculture applications, long-lived radionuclides associated with nuclear power installations, diverse metals and metalloids which may be associated with new emerging industries, and emerging pathogens that may arise from changing land use patterns and new forms of sewage treatment and disposal. The impacts of these new contaminants on coastal ecosystems and on human consumers are largely unstudied. Indeed, even the links between the best studied contaminants entering marine ecosystems and human health are only now starting to be drawn, and clearly there is much new research that is needed even for these contaminants.

The Mediterranean and Black Sea are ringed by densely populated coastal communities that are often in a state of flux in sheer numbers, in socio-economic development and in agricultural and industrial activity. Moreover, river diversion in some areas, for transportation, hydroelectric power, and irrigation purposes can greatly influence the entry of contaminants and pathogens into estuarine waters. Increasingly we are recognizing that industrial shipping and pleasure craft can also transport toxic microorganisms from one region to another. Different geographic regions are often facing similar types of environmental and public health problems, and yet there are relatively few coordinated efforts to address these problems or even summaries of the current state of knowledge in these areas that managers or scientists can be guided by.

To assess the scientific dimension of such challenges, CIESM organized a Workshop in May 2004 in Neuchatel. In welcoming the participants Frédéric Briand expressed his gratitude to Nicholas Fisher and Gerhard Herndl, Presidents respectively of the Committees on Marine Biogeochemistry and Marine Microbiology, who had suggested the central theme and agreed to co-chair this event, and to François Nyffeler, Representative of Switzerland on the Commission, for his efficient assistance on the logistic side. Thirteen scientists from ten countries attended the brainstorming sessions at the invitation of CIESM. This summary - a synthesis of the discussions and of follow-up consultations within the group – must be considered essentially as a team effort.

2. NOVEL CHEMICAL CONTAMINANTS

2.1 Contaminants of concern

The history of investigations and discussions on chemical pollutants now covers more than 40 years. Major political and scientific milestones were Rachel Carson's book "Silent Spring" in 1962, James Lovelock's Gaia Hypothesis published in 1989, the book entitled "Our Stolen Future" by Theo Colburn, and the Stockholm Persistent Organic Compounds (POP) Convention adopted on May 22, 2001. The latter encompasses 12 polychlorinated chemicals (hydrocarbons, biphenyls, benzodioxins and benzofurans), the so-called dirty dozen and have become legally binding on May 17, 2004 following the ratification by more than 55 countries.

In the last two decades environmental scientists provided evidence for the spread throughout the global environment of certain persistent organic compounds (such as many chlorinated hydrocarbon compounds) and quantitative understanding of the mechanisms of the distribution of these and other compounds in environmental reservoirs, including living organisms and human populations. The scientific community also has developed an expanded data base on the biochemical reactivity and potential for biological effects (e.g. immunotoxicity, endocrine disruption and carcinogenicity) of various organic pollutants in animals - including humans. Recently, for instance, the endocrine disrupting effects of some organic contaminants have been recognized as having great potential for detrimentally affecting wildlife.

The "conventional" contaminants of the 1960s to 80's have been eclipsed by a long list of "potential bad actors." Compounds like PCBs, PAHs, and organochlorine pesticides are well-investigated, while environmental data for emerging contaminants such as pharmaceuticals (e.g., analgesics, blood-lipid regulators, beta-blockers, anti-epileptics, X-ray contrast media), antibiotics (e.g. fluoroquinolones, macrolides, sulfonamides, tetracyclines), steroidal hormones, flame retardants (e.g., polybrominated diphenyl ethers, hexabromocylododecane, tetrabromobisphenol A, organophosphorous flame retardants), polyfluorinated surfactants, and constituents of personal care products are rather rare. The paradigm of cancer causing compounds, such as PAHs and PCBs, has been supplemented to contaminants that cause endocrine disruption, act as "gender benders" or trigger microbial resistance. Thus, "New Environmental Quality" standards are evolving that are linked to the analytical determination of many of these new polar contaminants. A classification of classic vs. novel contaminants in relation to their environmentally relevant physicochemical properties is attempted in Figure 1. Table 1 presents some information on classification and environmental behavior of novel chemical contaminants that enter coastal waters.

Table 1. Incomplete overview on currently emerging contaminants.

Types	Chemical compound classes	Current environmental knowledge	Chapters of this report* and literature
Pharmaceuticals and metabolites	Broad range of chemical composition	Wide input via municipal wastewaters	...
		and hospital effluents	Kolpin *et al.*, 2002
		As above, plus aquaculture	Heberer *et al.*, 2002
			Sacher *et al.*, 2001
			Snyder *et al.*, 2003
Antibiotics	fluoroquinolones		Giger *et al.**
	macrolides		Giger *et al.*, 2003
	sulfonamides		Golet *et al.*, 2003
Endocrine disruptors	17β-estradiol, ethinylestradiol, bisphenol A, nonylphenol	Wide input via municipal wastewaters	.....
Flame retardants	Polybrominated diphenylethers (PBDEs)	Ubiquitous POPs	Hites, 2004
	Hexabromocyclocdodecane (HBCD)	atmospheric transport	
	Tetrabromobisphenol A (TBBPA)		
	organophosphates		
Polyfluorinated surfactants	perfluorooctane sulfonate (PFOS)	New POPs, unclear input	Schultz *et al.*, 2003
	perfluorooctanoic acid (PFOA)		
Polar pesticides and metabolites			
Nonagricultural biocides	organotins, irgarol, dichlorooctyl-isothiazolinone	Antifungal chemicals for boats.	
	triclosan	Biocidal products	
Additives			
Plasticizers	Phthalates, organophosphates		
anticorrosives	benzotriazoles		
preservatives	isothiazolinones		
	polychlorinated paraffins		Marvin *et al.*, 2003
Surfactants and metabolites	nonlyphenolpolyethoxylates, nonylphenol	EU risk assessment report for NP	Ahel and Terzic*
			Stephanou*
			Montgomery and Reinhard, 2003
Gasoline additives, oxygenates	MTBE		Deeb *et al.*, 2003

The identification and determination of concentrations of many previously undetected organic anthropogenic compounds in the environment has progressed rapidly in recent years with the development of new and improved analytical techniques. Furthermore, it has been found, that the detection of certain of these "novel" contaminants such as polybrominated diphenyl ethers (PBDEs); perfluorochemicals: perflurooctane sulfonates (PFOS) and perfluorooctanoic acid (PFOA); alkylphenolic compounds – nonyl- and octylphenol; many pesticides (e.g., triazine and phenylurea herbicides); veterinary and human pharmaceuticals; biocides and bactericides (e.g., irgarol and triclosane); and phthalate esters, among others might be of environmental concern, because they have been shown to be mobile, persistent and toxic and some are bioaccumulative. Studies have also shown that the levels of at least some of these chemicals have increased in recent decades and that their presence in the environment is widespread (e.g., PBDEs).

Exposure assessment in Europe is hampered by the lack of monitoring data for the above-mentioned compounds. In addition, wastewater is targeted in some Mediterranean regions with an important lack of water resources, a susceptible aquatic environment and the important need of water re-use. The presence and the behavior of these compounds in sewage treatment plants will determine their occurrence not only in Mediterranean fresh waters (rivers, groundwater, etc.) but also in the marine environment, and possibly in the coastal atmosphere.

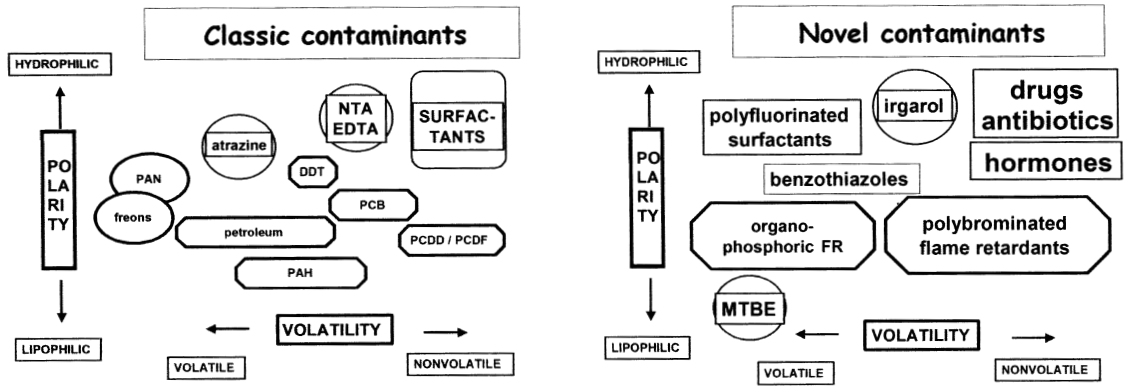

Fig. 1. Comparison of "classic" and "novel" contaminants with respect to polarity and volatility.

2.2 Analytical methods for measuring novel contaminants

A sophisticated operation of instruments and data evaluation is a prerequisite for successful analysis of emerging contaminants. Solid-phase extraction in combination with liquid chromatography (LC) on reversed phase columns have been mostly used for sample pre-concentration and for compound classes like pharmaceuticals or estrogens. The introduction of liquid chromatography directly coupled to mass spectrometry (LC/MS) has enabled the detection and quantitative determination of trace amounts of more polar organic contaminants. Electrospray ionization (ESI) and atmospheric pressure ionization (APCI) are the most frequently used ionization techniques for polar and ionic compounds, as well as for less polar nonionic ones. Quantitative assessment of the environmental occurrence and behavior of these emerging contaminants will require further development of sensitive analytical methods, based on the above mentioned techniques. Thus, the forefront analytical technologies that are suited for these mostly polar compounds are LC directly coupled to multistage mass spectrometry (MS^n), time-of-flight mass spectrometry (TOF-MS) and inductively coupled plasma mass spectrometry (ICP-MS).

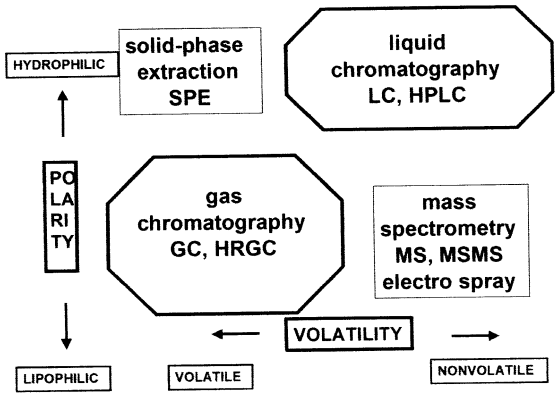

Fig. 2. Analytical approaches appropriate for analysis of novel chemical contaminants.

Multistage mass spectrometric techniques and the use of diagnostic ions reveal their usefulness for compound-class specific screening. In addition, several emerging contaminant classes can also be investigated directly by GC/MS without or after derivatization. An overview of the analytical techniques to be used for the environmental analysis of novel contaminants, in relation to the environmentally relevant physicochemical properties, is presented in Figure 2.

2.3 Sources of novel contaminants in coastal waters

As noted above, an increasing diversity of chemicals is entering the environment, including coastal waters. Sources include urban, industrial and agricultural areas or activities, and include direct discharges at point sources (such as water treatment plants and industrial waste waters) and more diffuse sources like runoff of drainage from agricultural areas. Discharges from some point sources, such as hospital waste waters entering urban sewage systems (Kimmerer, 2001), can present a unique suite of unusual contaminants. In addition, to land-based sources, the direct inputs of chemicals used for example in aquaculture should also be taken into account, most particularly certain types of pesticides (pyrethroids, for example) and antibiotics (see below).

The time course and frequency of inputs of these sources can vary considerably ranging from continuous emissions, seasonal uses and discharges, peak discharges and accidental events. Clearly, studies should take into account the spatial and temporal distribution of sources to allow better prediction and modeling of the distribution of these emerging chemicals in the environment.

Some specific examples of emerging contaminants in coastal waters can be given. Natural and synthetic steroid estrogen (e.g., 17a-ethinylestradiol) is present in sewage effluent and in receiving waters at levels that elicit biological effects such as endocrine disruption (Garcia-Reyero *et al*., 2001). Similarly Bisphenol A has been detected in wastewater effluents and sewage sludge (Furhacker *et al*., 2000). Phathalates have been used for more than 40 years in very large amounts for the manufacturing of different kind of plastics (e.g., PVC) and other resins (Fromme *et al*., 2002). DEHP has been detected in surface waters, sewage effluents and sediments (Watanabe *et al*., 1987). Antifouling agents such as TBT are used in marine paints on boats and vessels; marinas and shipyards are point sources for these contaminants in the marine environment. Surfactants are mainly used in detergents. The surfactants of most concern are the alkylphenols (AP) and their ethoxylates (APEO), linear alkylbenzene sulfonates (LAS), perfluorochemicals: perflurooctane sulfonates (PFOS) and perfluorooctanoic acid (PFOA), flame retardants and especially brominated compounds that have been used in plastics, textiles, electronic circuitry, and as chemical additives. This group includes polybrominated biphenyls (PBB), polybrominated diphenyl ethers (PBDEs), hexabromocyclododecan (HBCD) and tetrabromobisphenol A (TBBPA). Many diverse pesticides are used in agriculture as well as in urban and domestic settings. These include insecticides, herbicides, and fungicides. Pharmaceuticals, including human and veterinary drugs are highly variable and are used in large amounts. Prescription amounts for some high volume pharmaceuticals in Europe, e.g. anti-inflammatory agents or some antiepileptics, reach several hundred tons per year.

2.4 Environmental pathways, behavior and fates of novel contaminants

Many of these emerging chemical contaminants enter the environment by various pathways. Perhaps the most important inputs into coastal waters of novel contaminants are through riverine fluxes, where large drainage basins (e.g., Rhone, Po) contribute to their transport into the Western Mediterranean. In addition, the direct inputs of big urban centers near coastlines through sewage treatment plants and other non-point sources can also contribute to chemical loading in the coastal Mediterranean. Estuaries constitute an interface between continental drainage basins of the surface waters and coastal waters. They integrate fluxes from riverine basins and constitute transitional waters where dissolved and particulate chemical constituents are transported from continental to marine environments. Because estuaries and other coastal regions are often areas of active biological recruitment or nurseries, elevated concentrations of contaminants in these regions pose potential dangers to diverse populations far more than in open ocean waters. Moreover, these are areas of active harvesting of seafood by human consumers, hence the potential for public health impacts are more pronounced by contamination of estuaries than more

remote waters. Atmospheric fallout through dry and wet deposition could be important for certain compounds, but this route may be more likely to contribute contaminants to open waters.

Once in the marine environment many of these contaminants are mainly present in the water phase since they are medium to polar compounds. For a considerable number of novel contaminants, however, specific sorption mechanisms can lead to a significant mass transfer into sediments. Their solubility in water makes them mobile in the environment and enables their long transport via currents. They appear to be persistent in the environment, although detailed studies on their degradation rates in the environment at trace levels have not been undertaken.

Information about the occurrence and concentrations of novel contaminants in the coastal Mediterranean is still very limited. The occurrence of contemporary pesticides and antifouling agents has been reported in the coastal waters of the Mediterranean and other marine areas. The distribution and levels of selected compounds are summarized in this volume by Theobald, Zuccato, and Tronczynski. New data about the identification and concentrations of chemicals in river systems entering the Mediterranean provide evidence of the presence of diverse organic substances including pesticides, pharmaceuticals, surfactants and others that probably enter coastal regions of the Mediterranean. Further, more advanced data bases describing contamination of other marine environments (North Sea, Baltic Sea, coastal Atlantic Ocean) by previously undetected chemicals are now available. The concentrations of these emerging contaminants (e.g., pesticides, surfactants, antifouling agents) in the North and Baltic Seas indicate that large amounts of these chemicals are introduced into coastal waters, that the compounds are persistent, and their dissolved concentrations rise to levels that are higher than those of some "classic" contaminants (e.g., hexachlorocyclohexane). It is likely that processes leading to coastal contamination in other waters are also active in the Mediterranean. Indeed, human and veterinary pharmaceuticals have been detected at elevated levels (on the order of 10 to 100 ng L^{-1}), in Po river water, including one station at the mouth of the river (see Zuccato contribution, this volume), and levels and spatial and temporal distribution of antifouling agents and selected herbicides have also been reported for the coastal Mediterranean.

2.5 Bioavailability, bioaccumulation of novel contaminants

Clearly, concern about the introduction of contaminants of any type into marine waters stems from their possible danger to living organisms (including people). While we can measure the presence of contaminants in aquatic ecosystems, this does not indicate that these contaminants are influencing living organisms. Indeed, it is at present difficult to quantify the risks associated with many novel emerging contaminants, either to aquatic organisms or to human populations that consume seafood, because there is considerable uncertainty regarding the biological uptake and toxic effects of these contaminants in marine ecosystems.

As a first principle, it is well established that chemical contaminants can exert toxic effects on organisms only after they have been taken up; that is, contaminants that remain in water or bound to sediment, for example, and are not taken into living organisms can not be toxic (Newman, 1998). It is also known that contaminants may speciate chemically and physically in the environment in ways that affect the extent to which they are available for uptake by aquatic organisms (Campbell, 1995; organic reference). Therefore, the bioavailability of contaminants for aquatic organisms must be assessed in order to evaluate their bioaccumulation potential. Once a contaminant is associated with an organism it can exert toxicity (either lethal or sublethal) and/or it can be transferred to another trophic level where it may exert toxic effects. Such trophic transfer is well known for many inorganic (e.g., metals and metalloids) and organic (e.g., chlorinated hydrocarbons) contaminants (Fisher and Reinfelder, 1995). Thus, animals can obtain their contaminants both through dietary pathways as well as directly from the dissolved phase, whereas plants, which do not eat, only accumulate contaminants from the dissolved phase. The overall issue of metal and radionuclide bioaccumulation in marine organisms has been treated in a separate CIESM workshop (CIESM, 2002b), and many of the processes discussed in that document are relevant to other contaminants as well. Briefly, it can be stated here that kinetic models now exist to quantitatively distinguish the relative importance of solute vs. dietary sources of contaminants for aquatic animals (Landrum *et al*., 1992; Wang and Fisher, 1999a), and model predictions have been verified with independent field measurements for diverse

contaminants in marine, estuarine, and freshwater invertebrates and vertebrates (Wang *et al*., 1996; Fisher *et al*., 2000; Roditi *et al*., 2000; Baines *et al*., 2002). These models rely on measuring the assimilation efficiency of ingested contaminants and their efflux rates from the animals, and experimental protocols for measuring these parameters have been well developed (Fisher *et al*., 1996). These parameters have already been evaluated for diverse inorganic and organic contaminants in marine and freshwater organisms (Wang and Fisher, 1999b).

The extent to which contaminants get accumulated out of water into organisms can be quantitatively compared among contaminants and among organisms using bioconcentration factors. These values essentially reflect the extent to which contaminants are enriched in organisms relative to ambient water. Such bioconcentration factors are readily used in risk assessment models but complications arise with organic compounds, which can be metabolized by living organisms. Nevertheless, striking patterns have been demonstrated for measuring the bioaccumulation of many organic compounds across many types of organisms. Generally, it appears that the hydrophobicity of a compound, assessed as its octanol-water partition coefficient, can be related to the degree to which it is enriched in organisms (Schwarzenbach *et al*., 2003). Thus, for a compound whose biological uptake is not studied, knowledge of a compound's octanol-water partition coefficient (k_{ow}) may help in estimating the extent to which it is likely to bioconcentrate in organisms. However, for some of the ionizable compounds such as many of the pharmaceuticals released into the environment, log D (log K_{ow} adjusted for speciation to correct for ionized species) may be more useful as an organizing principle for comparing chemicals.

As noted above, once associated with an organism, a contaminant could exert toxicity either to that organism or to another organism that eats it. It appears that most contaminants in natural water do not reach sufficiently high concentrations to elicit acutely lethal effects. However, diverse sublethal effects are possible and might include diminished growth rates, impaired reproductive capability, altered metabolic pathways, and altered behavioral patterns in affected organisms. Such reduced fitness could have pronounced effects on the viability of populations in natural waters, leading to changes in community structure. It is widely acknowledged that sublethal toxicity tests need to be conducted to greatly expand the data base for effects on marine organisms, especially for contaminants that have only recently been measured in natural waters. Moreover, there is a need to improve toxicological testing protocols for aquatic organisms. For example, often the toxicity of a contaminant to a test animal is assessed by exposing the animal to dissolved contaminant, but of course animals (including man) can also accumulate contaminants through their diet, and the resulting tissue distribution of contaminants following different uptake pathways can be sufficiently different to greatly affect the biological response (Fisher and Hook, 2002). Thus, contaminant toxicity to animals also needs to consider dietary exposure.

Many contaminants associate with sediments, which have often been thought to be the final repository for particle-reactive metals and organic compounds. While estuarine sediments are often greatly enriched (relative to the water) in many contaminants, they can also be thought of as a source, not just sink, of contaminants for marine systems. There is now unequivocal evidence of biological uptake of diverse organic and inorganic contaminants bound to sediments by a wide variety of benthic invertebrates (Mayer *et al*., 1996; Griscom *et al*., 2000). These studies suggest that sediment-dwelling animals (both infaunal and epifaunal) can assimilate contaminants from ingested sediment as well as absorb contaminants from pore water and overlying water, and these animals may transfer contaminants to predators who consume them (Luoma and Fisher, 1997). The extent to which many of the emerging organic compounds get assimilated out of sediment into benthic animals and get subsequently transferred up the food chain has largely gone unstudied.

2.6 Biological effects of novel contaminants

Many of the novel contaminants studied in this report have endocrine disrupting properties, and are known to have carcinogenic, reprotoxic or neurotoxic effects on living organisms, often at extremely low concentrations. For example, among natural and synthetic steroid estrogens, ethinylestradiol (EE2) has been shown to induce vitellogin production in male rainbow trout at 0.1 ng l^{-1} levels (Purdom *et al*., 1994) and the growth and development of testes in maturing male trout has been shown to be retarded by 50% due to a single dose of EE2 at 2 ng l^{-1} (Jobling *et*

al., 1996) resulting in sex reversal in fish (Martin-Robichaud *et al*., 1994). Similarly, reproductive effects of Bisphenol A (BPA) have been observed on fathead minnow (*Pimephales promelas*), including inhibition of spermatogenesis (Sohoni *et al*., 2001). Still, BPA levels in surface water are typically one to several orders of magnitude lower than those which cause chronic effects in test organisms (Staples *et al*., 1998), although bioaccumulation in biota may occur), and the risk associated with contaminated sediment is likely to be much greater. Generally, the toxicity of phthalates to aquatic organisms increases with increasing alkyl chain length due to a corresponding increase in log Kow and decreasing aqueous solubility (Call *et al*., 2001); calculated predicted no effect concentrations (PNECs) for PAEs of varying alkyl chain length suggest that there is some concern for aquatic organisms and benthic species (Staples *et al*., 2000).

Tributyltin (TBT) residues provide one of the best examples of population level effects on wildlife (Van der Kraak, 1998). Both lethal and sub-lethal effects have been observed in biota (Voulvoulis, 1999), including severely retarded larval development, masculinization of females (imposex), behavioral changes and shell deformation in oysters. In some areas TBT exposure has resulted in sterility leading to species extinction (Minchin *et al*., 1996). Imposex has been determined at levels of 3 ng l^{-1} TBT, and a no effect concentration (NOEC) has yet to be identified (Burton and Scott, 1992). Generally, among surfactants, toxicity is greater to aquatic organisms than mammals, with toxicity for alkylphenolethoxylater (APEO) increasing with decreasing number of ethoxylate units and increasing hydrophobic chain length (Fent, 1995). A recent survey of wild roach in U.K. rivers has found a high percentage of males with eggs in their testes and female egg yolk protein in their blood attributed to exposure to such surfactants (Jobling *et al*., 1998). PAHs and their metabolites that are associated with sediment are bioavailable to both benthic and demersal aquatic organisms (Landrum *et al*., 1991), with the lifestyle of the organism affecting its exposure to sediment-associated PAH and the direct intake of particulates contributing a significant proportion (Woodhead *et al*., 1999).

Flame retardants and especially brominated compounds (PBDEs) are also of concern since they are persistent, lipophilic, have been shown to bioaccumulate (de Wit, 2002). Pesticides and other biocides are known to exhibit endocrine activity and have the potential to accumulate in the aquatic food chain due to their persistence and lipophilicity (Bulger *et al*., 1978; Ireland *et al*., 1980; Bedding *et al*., 1982; Bulger and Kupfer, 1983). There is much evidence linking organochlorine insecticide exposure to endocrine disrupting effects on the wildlife. Exposure results in both physiological abnormalities such as thinning of eggshells and damage to the male reproductive system, and to behavioral changes which are also potentially dangerous to survival (Colburn, 1995; LeBlanc, 1995). In addition, as a result of restrictions in the use of organotins in antifouling applications, alternatives to TBT paint such as copper based coatings containing organic booster biocides are of particular concern, because of limited data and information available on their occurrence, fate, toxicity, and persistence in the marine environment (Voulvoulis, 1999).

As a rule, there is currently little evidence to suggest that acute effects on aquatic systems are occurring. Exceptions can occur locally when untreated production or municipal waste is released directly into surface waters or estuaries. However, emerging contaminants such as pharmaceuticals may very well not be the most deleterious agents in these situations, where ammonia, toxic metals, and other anthropogenic, industrial or agricultural toxicants may control ecosystem health ("dead fish do not care for endocrine disruption"). There is, however, considerable scientific uncertainty regarding the environmental impact of pharmaceuticals due to their specific modes of action, the chronic exposure situation of aquatic life and the complex mixture situation (Seiler, 2002). Ecological effects data, taking into account chronic exposure and mixture effects, are being increasingly recognized (Cleuvers, 2004; Ferrari *et al*., 2003; Huggett, 2002; Ferrari, 2003; Laenge *et al*., 2001; Jones *et al*. in press).

An acute to chronic ratio (ACR) of 10-100 has been established for a large number of industrial chemicals. Some researchers claim now that for specific pharmaceutical classes, ACRs of 10,000 or higher might be more appropriate. For specifically acting substances (like many pesticides or pharmaceuticals) a recent review showed a median ACR of 10.6, with a minimum-maximum

range of 1.33 – 34800 and a 95th percentile ACR of 191 (ECETOC, 2003). For comparison, a few available examples have been compiled below and the respective ACRs have been calculated.

Table 2. Acute to chronic ratios (ACRs) in vertebrates for a selection of human pharmaceuticals.

Substance	**Acute data**		**Chronic data**		**ACR**	**Ref. (chronic data)**
	Type	Value	Type	Value		
Ethinylestradiol	LC_{50}	1.5 mg L^{-1}	Chronic NOEC	1 ng L^{-1}	1.5 x 10^6	Laenge *et al.*, 2001
Estradiol	LC_{50}	6.1 mg L^{-1}	Chronic EC_{50}	0.12 µg L^{-1}	50,833	Kramer *et al.*, 1998
Propranolol	LC_{50}	24.3 mg L^{-1}	Chronic LOEC	0.5 µg L^{-1}	48,600	Huggett *et al.*, 2002
Fadrozol	LC_{50}	49 mg L^{-1}	Chronic LOEC	2 µg L^{-1}	18,500	Ankley *et al.*, 2002
Carbamazepine	LC_{50}	43 mg L^{-1}	Chronic NOEC	25 µg L^{-1}	1,720	Ferrari *et al.*, 2003
Diclofenac	LC_{50}	50 mg L^{-1}	Chronic NOEC	1 mg L^{-1}	50	Ferrari *et al.*, 2003
Thiabendazole	LC_{50}	0.56 mg L^{-1}	Chronic NOEC	0.012 mg L^{-1}	46	EPA online data [a]

[a] http://www.epa.gov/pesticides/reregistration/thiabendazole/

Contaminants do not reach the environment as individual chemicals but are present in complex and constantly changing mixtures. Current RA procedures do leave this aspect largely out of consideration. In the aquatic environment most organisms are continually exposed to numerous potentially toxic substances simultaneously with possibly only slight temporal and spatial variations in concentration levels (Schowanek, 1998). Recent work is beginning to demonstrate the significance of exposures to mixtures of chemical (and non chemical) stressors at low concentrations and this raises the concern whether additive effects might occur or whether synergy could magnify the effects of certain pharmaceuticals (Fox, 2001; Renner, 2002). Most available experimental evidence suggests concentration additivity as opposed to synergistic or antagonistic effects, but there is still a lot of work to do in this area.

2.7 Antibiotic resistance

Antibiotics are chemicals that have received considerable attention in recent years, in part because of potential public health concerns, and in part because of their known abundance in coastal waters from aquaculture operations as well as sewage inflow. Salmon farming uses large amounts of antibiotics to keep disease and parasite outbreak under control. Recently, antibiotic use has gone down considerably in some countries, while vaccinations have increased. Norway, for example - once considered a major user of antibiotics on fish farms - the consumption of antibiotics has been reduced by 96% in the course of the last ten years because the country vaccinates now every single farmed salmon. (Figure 3, source: www.fiskeoppdrett.no/akvakultur/html/Aquaculture.htm).

Some of the more common antibiotics that are used in aquaculture today include oxytetracycline and amoxycillin. The antibiotics are delivered to the target species in the form of medicated feed products. However, not all the feed is consumed, and hence some of the antibiotics are released into the environment, and often are transported into the sediments (Herwig *et al.*, 1997; Pouliquen and Le Bris, 1996; Munekage *et al.*, 2002). As a variety of antibiotics are used in both human medicine as well as aquaculture practices, their presence in the environment is of concern due to the possibility of increased bacterial drug resistance. The persistence of several antibiotics in marine sediments has been tested by Hektoen *et al.* (1995), and it was found that particularly oxytetracycline, as well as several quinolones (oxolinic acid and sarafloxacin), tend to be rather resistant (on the order of months) within sedi-

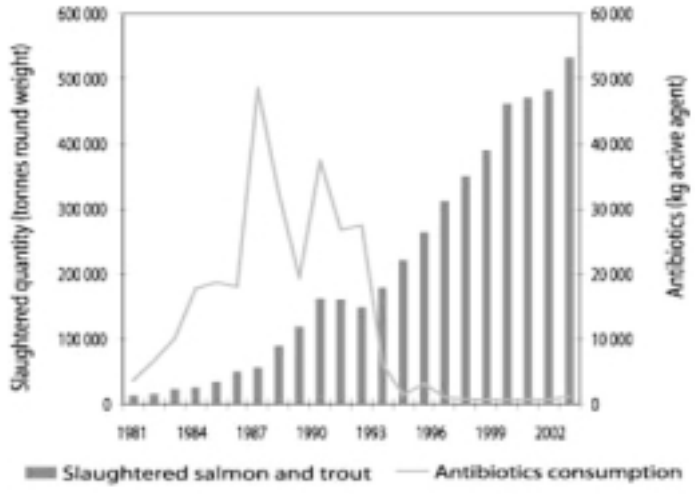

Fig. 3. Antibiotics use in relation to salmon and trout farms since 1981.

ments. Resistance of sediment bacterial populations to tetracycline has been investigated in a sturgeon farm by Forney *et al*. (1998). Their results indicated that antibiotic concentrations higher than the recommended therapeutic levels are necessary in order to produce a noticeable effect on the bacterial populations within the sediments. However, Herwig *et al*. (1997) found that antibiotic resistance in bacteria within the sediments increased in close proximity to salmon net cages in Puget Sound, WA, U.S.A. Furthermore, it is assumed that the extensive use of antibiotics within the shrimp industry in Ecuador may have resulted in the occurrence of the antibiotic-resistant *Vibrio cholerae* in humans (MacMillan, 2001). In addition, bacterial resistance to another type of antibiotic, flumequin, can be high so that only about 10% of the bacteria found in intensive aquaculture systems are unable to grow in its presence (Migliore *et al*., 2001). Another study by Schmidt *et al*. (2000) demonstrated the impact of trout farming on environmental bacteria as well as fish pathogens. Their findings show that antibiotic resistance levels were increased in those populations (especially those composed of aeromonads and Flavobacteria) that were exposed to the antibiotics.

Mediterranean concentrations of antibiotics, vaccines, and other chemotherapeutants have only been sporadically studied. In a study of coastal sediments in the Ligurian Sea (Western Mediterranean), Chelossi *et al*. (2003) found a threefold increase in bacterial density and biomass in sediments beneath the fish cages compared to control sediments. Antibiotic sensitivity tests showed a high percentage of resistant strains in both control and impacted sediments, but ampicillin resistance and multiple resistance patterns in bacterial isolates was greater in impacted sediments.

2.8 Risk assessment for novel chemicals

Environmental risk assessments for environmental contaminants are based on tiered decision trees with a constantly increasing level of detail and data. For pesticides and industrial chemicals, guidelines are quite detailed, however, for human pharmaceuticals, the proposed approach is quite simple (Straub, 2002). For human pharmaceuticals, as a prioritization tool, predicted exposure levels (PECs) are used prior to entering Tier 1. This is based on tonnage or use worst-case exposure estimation, refined by various depletion mechanisms such as metabolism or biodegradation. Effect assessment (PNECs, or predicted no-effects concentrations) is based on acute effects data in selected predefined species (e.g., algae, daphnia and fish). A next higher tier with a refined fate or effect assessment has to be entered if the risk ratio in an earlier tier indicates a concern (e.g., PEC/PNEC >1). While being an adequate screening tool, the approach has been criticized due to problems associated with the lack of data required for PEC and PNEC estimation, chronic toxicity data, and mixture effects.

In order to be able to properly assess the risks associated with the sources, use and disposal of these chemicals, several knowledge gaps need to be filled. These include data on:

- Sources
  - Tonnage (production)
  - Usage patterns
  - Disposal
- Behavior in relevant environmental compartments
  - Sewage Treatment Plants (STPs)
  - landfills
  - soils / runoff
- Exposure
  - Levels
  - Persistence
  - Bioaccumulation / Biomagnification
- Effects
  - Acute Toxicity
  - Chronic Toxicity

For conventional chemicals, where these data are often available, risks have been established and in a few cases, mitigation measures have even been introduced to reduce impacts (e.g., DDT, CFCs, lead in gasoline). For novel contaminants, despite considerable concern associated with some of them (e.g., estrogens), uncertainty is still too high because of the lack of data, to allow risk management strategies to be established. Moreover, for all classes of contaminants, sublethal toxicity testing with marine organisms needs to be improved, and must take into consideration the accumulation of contaminants in tissues (that is, effects should be expressed as a function of dose in the organism, not outside the organism) as well as toxic effects of contaminants accumulated through different uptake pathways.

2.9 Prioritizing, ranking of chemicals

Under Directive 2000/60/EC of the European Community, specific measures were decided at the Community level against pollution of water by individual pollutants or groups of pollutants presenting a significant risk to aquatic environment. Such measures are aimed at the progressive reduction, and for priority hazardous substances phasing out, of discharges, emissions and losses into environment of these substances. The ultimate aim for the marine environment is to achieve concentrations approaching background values for naturally occurring substances and close to zero for man-made substances. With a view to the adoption of these measures the European Commission has introduced a scientifically based methodology for selecting priority substances on the basis of their significant risk to or via the aquatic environment. This methodology includes the application of a simplified risk-based assessment procedure based on scientific principles taking into account:

(1) evidence regarding the intrinsic hazard of the substance concerned, and, in particular, its aquatic ecotoxicity and human toxicity via aquatic exposures routes;

(2) evidence from monitoring of widespread environmental contamination;

(3) other proven factors which may indicate the possibility of widespread environmental contaminantion, such as production, use volume and use pattern of the substance concerned.

Table 3. Sale data of pharmaceuticals in 2001 (based on prescriptions in Italy). From Calamari *et al.* (2003).

Pharmaceuticals (Therapeutic categories)	**Sales in year 2001 (kg per million people)**
amoxycillin (antibacterial)	3680
ceftriaxone (antibacterial)	150
ciprofloxacin (antibacterial)	260
clarithromycin (antibacterial)	590
erythromycin (antibacterial)	72
spiramycin (antibacterial)	91
lincomycin (antibacterial)	130
atenolol (β - blocker)	390
bezafibrate (lipid regulating)	130
Enalapril (antihypertensive)	86
furosemide (diuretic)	18
hydrochlorothiazide (diuretic)	257
omeprazole (ulcer healing)	59
ranitidine (ulcer healing)	467

The Commission has on this basis, developed a combined monitoring-based and modeling-based priority setting (COMMPS) scheme. The identification of priority hazardous substances should be made with regard to hazardous substances agreed for phase-out or for cessation of discharges, emissions and losses in international agreements such as the UN-ECE and OSPAR Convention, including hazardous substances identified by OSPAR DYNAMEC Selection (I and III) of substances that are persistent.

Estimate of pharmaceutical use provide some information as to the likeliest compounds that may reach coastal waters. The five leading therapeutic classes in 2000 according to IMS were antiulcerants, cholesterol lowering agents, antidepressants, blood pressure lowering agents and Ca^{++} antagonists. While detailed tonnage data are difficult to obtain, it

can be assumed that the tonnage range of high volume pharmaceuticals can reach several hundred tons annually worldwide. Another example of pharmaceutical use can be inferred from the sales figures for diverse compounds in Italy (Table 3).

3. PATHOGENS IN COASTAL ECOSYSTEMS

Global incidence of many water born infectious diseases is rising due to increased prevalence of existing pathogens and the emergence of novel pathogenic organisms. These emerging infectious diseases most likely result from a combination of environmental changes such as eutrophication, deforestation and climate change, as well as specific human behavior such as increased globalization and excessive use of antibiotics in agriculture and aquaculture (Rocourt *et al*., 2003; Theron and Cloete, 2002; Harvell *et al*., 2002; Ruiz *et al*., 2000).

In the Mediterranean Sea, global warming is expected to result in a spreading of subtropical species, including pathogens, into the Mediterranean climate zone. Desertification has also been identified as one of the key climatological problems that some Mediterranean countries will face in the coming decades, with accompanying changes in the terrestrial and freshwater biota. Pathogens originating from other seas may be introduced into the Mediterranean Sea with ballast water discharge by ships (Ruiz *et al*., 2000; CIESM, 2002c). Human demographics are changing as well. With rapidly increasing human populations along the Mediterranean coastline, many cities and towns still do not have sufficient sewage treatment. Along with increasing human populations, agricultural practices have been linked to increased incidences of outbreaks of classical waterborne disease as well as the development of new virulent strains of bacteria and viruses (Table 4). We are not aware of epidemiological data published from the Mediterranean region.

In response to these developments, there is a pressing need for 1) better risk assessment and improved monitoring programs for waterborne pathogens, 2) a better understanding of waterborne pathogens as components of aquatic ecosystems to help improve our understanding of their distribution and dynamics and 3) increased use of molecular technologies in monitoring and environmental research of waterborne pathogens.

Table 4. Incidence rate of notified diseases in developed countries (per 100,000 of human population).

Notified disease	Incidence rate /100 000 pop/year	Reference
Viral gastroenteritis	28 000	DeWit 2001
Gastroenteritis (norovirus)	14 000	Lopman 2002
Campylobacteriosis	100	"Foodborne" 1997
Salmonellosis	32	"Foodborne" 1997
Hepatitis A	12	"Foodborne" 1997
Shigellosis	5.6	"Foodborne" 1997
Vibriosis	2.5*	"Risk assessment" 2002
Yersiniosis	2.2	"Foodborne" 1997
Typhoid fever	0.5	"Foodborne" 1997
Listeriosis	0.4	Anon. 2002

* estimation from US data.

3.1 Better risk assessment

Monitoring programs currently practiced by Mediterranean countries test for *Escherichia coli* in seafood, drinking water, and waters used for recreational purposes (EU directive). Other bacterial pathogens such as *Vibrio cholerae, V. parahaemolyticus,* and *Shigella* are only searched for after outbreak incidents. In addition, sampling for viral pathogens is not yet routine (Butt *et al*., 2004; Wyn-Jones and Sellwood, 2001; Bosch, 1998).

The use of *E. coli*, as a reference indicator or culture techniques to detect pathogens, as presently done, fails to reduce risk. For example, shellfish responding to EU criteria (< 230 *E. coli*/100mg) were found to be responsible for significant outbreaks linked to shellfish consumption (Butt *et al*., 2004). Recommendations to improve monitoring efforts are:

- Include more major pathogens in routine sampling (Table 3).
- Couple risk assessment with the development and application of new molecular tools as proposed by Rose and Grimes (2001) that could provide a significant advance in protecting consumers.
- Reinforce the human population surveys for pathogens, especially in response to outbreaks in the populations close to beach or shellfish production areas. This will allow better prioritization of which pathogens to monitor depending on the epidemiological status of the coastal human population.
- Develop and support Mediterranean-wide data bases containing all the information on circulating strains, serotypes, emergence of mutants. Data base coordination and co-ordination of North and South shore countries is currently non-existent, but could be especially critical with emerging pathogens.
- Implement early warning systems to currently assess the water quality in bathing or seafood harvesting areas. This system would combine currently available data potentially relating to water quality degradation (i.e., rainfall events, real time monitoring of sewage treatment plants or sewage network failures, variations in river flow), with data from probes implemented in the environment (shellfish beds or buoys) able to provide real-time data on salinity, chlorophyll, pH. The data could be provided to central communication networks to model and predict the wastewater inflow and its impact on water quality. Combined with spatial information on pathogen outbreaks, this would allow predictions of pathogen concentrations in the water on a site-specific basis. Modeling the impact of possible events could also improve our understanding of the effects of sewage treatment plants and rivers in affecting water quality. This early warning system, if implemented, would be validated by sampling trials, for example to establish a link between decreased salinity and the presence of fecal pollution and pathogens.

It is of interest that a new Mediterranean research project (CHOLCLIM) has been established for the quantification of emerging risks of cholera outbreaks in the Mediterranean basin in relation to climate change using spatial teledetection and epidemiological modeling. Headed by JF Gueguan, IRD, Centre de Montpellier, this project is based on observations of recent outbreaks on Mediterranean coasts in Italy, Greece, Turkey and Spain. The objectives are (1) to analyze the factors that contribute to outbreaks using information from satellites, modeling, and physical and biological oceanography, and (2) to propose different scenarios to assess and manage future outbreak risk. Scientific partners involved in the project are CNRS, IRD, Medias-France, IPLS, LMD, Univ. Princeton, Univ. Cambridge). For more information concerning the project and others on climate change, see htpp://medias.obs-mip.fr/gicc.

3.2 Considering pathogens in an ecological context

Waterborne bacterial and viral pathogens do not just interact with human hosts. They are also integral elements of the aquatic environment. Some human pathogens are naturally occurring organisms in aquatic ecosystems. For example, *Vibrio* spp., several of which are human pathogens, persist in aquatic ecosystems indefinitely as members of the bacterioplankton and are subject to various factors that impact the dynamics of plankton communities (e.g., Cottingham *et al*., 2003). Other pathogens may exist outside the human host only in a transition stage, requiring input from infected humans to remain persistent in aquatic environments, but the spatial and temporal distribution of these elements will still be influenced by ecological parameters.

A better understanding of the ecology of human pathogens can lead to development of better risk assessment programs and may elucidate ecosystem management or human behavior options that reduce the risk of disease outbreaks. Environmental parameters such as temperature, salinity and nutrients, as well as interactions with other aquatic biota will affect the seasonal and spatial distribution and virulence of these pathogens. This will in turn affect when, where and how often humans will be at risk. For example, factors that affect filtering rates and lipid accumulation in shellfish will affect their uptake and retention of viral particles, which in turn can influence the risk of outbreaks from shellfish consumption (Rose and Sobsey, 1993; Lee and Morgan, 2003).

Of particular interest in the field of emerging and novel pathogens is a greater understanding of environmental conditions that may lead to the development of more virulent pathogen strains.

Understanding the links between aquatic reservoirs of pathogens (sewage treatment plants, lakes and rivers, estuaries, sediments, coastal ocean) is another crucial facet of disease ecology. Pathogens introduced into the Mediterranean originate largely from point sources such as rivers due to insufficient wastewater treatment. Thus, the distribution of pathogens is likely to be patchy and tightly linked to the overall hydrography of the coastal systems receiving the input.

Characteristic Mediterranean subsystems that are especially vulnerable to accumulation of pathogens are lagoonal systems. These typically have long water residence times accompanied by high receiving loads of wastewater and a generally shallow water column. High levels of biotic and abiotic particulates in these shallow systems might cause rapid settlement of pathogens via adsorption or active attachment and subsequent sedimentation. Coastal sediments might therefore act as temporary depositories of pathogens, becoming potentially mobilized again upon sediment resuspension.

3.3 Development and application of molecular technologies

Monitoring for many pathogens is still primarily done by culturing approaches (Moe, 2002; WHO, 2003). A well-documented drawback of this technique is that these pathogens are often not in a culturable state and will therefore escape detection (McDougald *et al*., 1998; Huq *et al*., 2000; Hot *et al*., 2003). Indicator pathogens such as *E. coli* are also often used to assess risk from other pathogens, but there is often poor correlation between indicator organisms and risk from other target pathogens (Toze *et al*., 1999; Hot *et al*., 2003; Butt *et al*., 2004).

Molecular techniques are increasingly applied to improve our ability to directly detect abundance of viral, bacterial and protozoan pathogens in water and wastewater. Often, these methods have the added advantage that they can potentially provide information about the viability, metabolic activity and virulence of pathogens *in situ*. These new methodologies are not yet widely used and most are still in developmental stages (Toze, 1999; Rose and Grimes, 2001; Theron and Cloete, 2002; Brinkman *et al*., 2003; Call *et al*., 2003; Straub and Chandler, 2003; Gruden *et al*., 2004). It is important for researchers and public health workers to be aware of these tools as they become available and to incorporate them into their monitoring and research practices whenever possible.

Fluorescent antibodies and molecular markers unique to a particular pathogen or even a particular strain of that pathogen can be used in conjunction with microscopy or, more efficiently, flow cytometry to directly assess pathogen abundance and viability in water samples (Huq *et al*., 1990, 2000; Theron and Cloete, 2002; Gruden *et al*., 2004). The transcription of specific genes may be indicative of the presence, metabolic activity, virulence or transmission risk of a pathogen. If these genes are unique to the pathogen in question, PCR (polymerase chain reaction), RT-PCR (reverse transcriptase-PCR) or QPCR (quantitative PCR; Brinkman *et al*., 2003) could be used to obtain qualitative or quantitative indications of pathogen abundance or activity from environmental and seafood samples. Microarray chips can simultaneously measure transcription of hundreds or thousands of genes to study whole genome response of a pathogen under environmental or experimental conditions. This approach may soon be adapted for pathogen monitoring by developing gene chips that can measure transcription products from multiple selected indicator genes in single pathogens or multiple pathogens (Rose and Grimes, 2001; Call *et al*., 2003).

Molecular approaches can also be applied to studying pathogen ecology. The genomes of many pathogens have been sequenced, and whole genome RNA transcription can be measured using microarrays. For many pathogens, the functions of myriad genes and genetic regulatory systems have been well studied in a medical context, but their activity and functions in aquatic environments are unknown. This makes microarrays a particularly powerful tool for understanding the detailed molecular response of pathogens to a wide range of environmental parameters and habitats. A more focused approach is also possible, using more specific microarrays or PCR techniques to follow expression of genes or protein products of particular interest. Finally, mutants can be constructed to examine the role of specific pathogen phenotypes in environmental growth and survival (such as attachment to plankton by *V. cholerae*; Chiavelli *et al*., 2001, this volume).

Major limitations to expanding monitoring programs and to implementing comprehensive research programs are the time and effort required to process samples, and the consistency and reliability of methodologies. A significant advance is the current development and testing of flow cytometry and multiplex-PCR methodologies capable of simultaneously and rapidly detecting multiple pathogens (Kong *et al*., 2002; Brinkman *et al*., 2003; Lee *et al*., 2003; Gruden *et al*., 2004). Ideally these methods could be developed into inexpensive, commercially available kits capable of high-throughput processing of either solid (e.g., sediments, shellfish) or liquid samples (Straub and Chandler, 2003).

3.4 Conceptual and practical links between novel contaminant and pathogen studies

Researchers and health workers involved in studying novel contaminants in Mediterranean systems would benefit from collaborating with those studying emerging pathogens and vice versa as there are a number of clear links between the fields. One of the most obvious link is sewage treatment plants, which can serve as the point sources for both chemical and pathogenic "contaminants" into aquatic ecosystems. Consequently, hydrological events could have similar effects on the distribution of both chemical contaminants and pathogens. Another particular concern with sewage treatment plants is that the co-occurrence in high concentrations of mutagenic and antibiotic contaminants and pathogens is very likely to be a potential contributor to emergence of new or resistant pathogen strains. Likewise, excessive use of antibiotics in aquaculture provides another potential direct link between novel contaminants and emerging pathogens and deserves further investigation.

There is a lack of comprehensive baseline monitoring programs and of research linking environmental parameters with abundance and distribution for both novel contaminants and pathogens in the Mediterranean. Given the expense of implementing effective monitoring and research programs, combined sampling efforts for waterborne contaminants and pathogens could be highly beneficial.

Finally, the molecular approaches available for studying pathogenic microorganisms can be applied to better understand sublethal biological impacts of contaminants in both laboratory and in situ studies (Purohit *et al*., 2003). Many aquatic pathogens have far more well-studied genomes than typical indicator organisms used in ecotoxicology. For example, genetic regulatory systems involved in such processes as cell division and growth, nutrient uptake, membrane function, antibiotic resistance, etc. are relatively well understood, making such organisms, which are also natural members of planktonic and sedimentary communities, potentially very useful in studying specific sublethal effects of novel contaminants. The same molecular techniques (PCR, microarrays, etc.) that can be used for specific environmental monitoring and assessing a pathogen's response to environmental parameters can be used to assess the response of these microorganisms to different types, concentrations, and combinations of contaminants.

Of course this approach is not limited to pathogenic microbes. The number of organisms with sequenced genomes is rapidly increasing. In addition to pathogens, genomes are now known or being obtained for a number of traditional ecotoxicology indicator organisms in other taxonomic groups such as the green alga *Chlamydomonas* and the microcrustacean *Daphnia*. Functional genetic information is typically less complete for these organisms than for pathogenic microorganisms, but this situation is changing rapidly.

4. Recommendations for Future Research

Analysis of novel contaminants

Analytical methods, including compound enrichment, separation and determination, should aim at limits of quantification at the level of ng L^{-1}. Enrichment techniques, such as solid phase extraction and semi-permeable membrane devices have to be improved with respect to their selectivity for polar compounds. TOF-MS for accurate identification, MS^n, and ICP-MS have to be optimized for polar analytes. Multistage mass spectrometric techniques and the use of diagnostic ions should be explored for compound-class specific screening to explore their usefulness for precise quantification of many novel contaminant compound classes, particularly in complex matrices such as marine sediments and sludge. Although LC directly coupled to the above mentioned mass spectrometric techniques is the best choice for polar contaminants, the use

GC/MS-MS and high-resolution mass spectrometry are useful techniques for some compounds classes such as brominated flame retardants. In addition, GC/MS-MS is still a powerful tool for the identification of unknown compounds. The spectra data bases for LC-MS/MS (APCI, ESI modes) should be further improved and made available for specialized laboratories. Due to the specific analytical techniques necessary and QA procedures, a joint cooperation of specialized laboratories is recommended.

The coupling of the quantifiable bioeffects of the species to be determined with their molecular structure will allow us to confront the challenging question of the relevance of the environmental analytical data obtained.

Environmental behavior of novel contaminants

Because of the considerable lack of field data in the Mediterranean Sea it is recommended that surveys must be conducted in selected coastal areas of the most important rivers entering the Mediterranean Sea to measure the concentrations of target compounds identified in this volume. Special attention should be paid to estuaries (especially those of the largest rivers), delta regions and lagoons, which are often close to areas of intensive human activity. The systematic monitoring of most relevant novel contaminants in the marine environment should be started as early as possible to establish appropriate baseline data and allow for estimates of temporal changes in coastal contamination. It is further recommended that joint sampling ventures and common analytical protocols be established between cooperating laboratories from different nations. CIESM may play an important federating role in this sector.

Biological Uptake and Effects of novel contaminants

(1) For those chemical contaminants identified as being present at above trace concentrations (ng/L) in water or sediment, biological uptake experiments should be conducted to measure their potential bioaccumulation. Experiments should focus on prominent taxonomic components of coastal waters, including phytoplankton, invertebrates, and possibly fish. Consideration should be given to using mussels as bioindicators of coastal contamination by emerging contaminants (much as they are used with conventional contaminants) to discern spatial and temporal patterns of bioavailable contaminant concentrations. CIESM Workshop Monograph No. 15 (2002a) describes a proposed network using mussels to assess spatial and temporal trends in coastal contamination by radionuclides in the Mediterranean, and inclusion of mussel samples for novel contaminant analyses could be coordinated with this ongoing effort. Biological uptake studies should be conducted to enable the application of kinetic models of contaminant uptake.

(2) Develop a classification /grouping approach for novel contaminants that exhibit the same mode of action and are structurally similar, e.g. pharmaceuticals, based on mechanisms of action.

(3) Given the unlikelihood of acute toxicity of the emerging chemical contaminants at environmentally realistic concentrations, toxicity tests should focus on sublethal or chronic effects. For many of the emerging contaminants, particular attention should be paid to consider endocrine disruption that may reduce the fitness of an individual organism and that could lead to a decline in a population. Toxicological assessments should primarily consider those contaminants which display bioaccumulation. Toxicity experiments involving animals should consider dietary as well as solute exposure. Since coastal waters are often contaminated with many co-occurring chemicals, some rational basis to evaluate the effects of mixtures of contaminants, in addition to the effects of individual compounds, should be attempted.

(4) For those radioisotopes that are released by hospitals that enter coastal waters via sewage treatment plants, emphasis should be placed on isotopes of elements that are known to accumulate in marine organisms. The radioactivity accumulated should be placed into the context of the natural radiation background experienced by those organisms (see Rose and Fisher, this volume).

Pathogens

(1) To improve risk assessments, looking for the pathogens potentially present in the area according to epidemiological information from the country;

(2) To develop and apply molecular techniques to directly detect pathogens;

(3) To implement an early warning system to mitigate against events (rainfall, sewage treatment plant failures, outbreaks, etc.) that may impact water quality and thus provide available tools for risk management.

5. TARGETED RECOMMENDATIONS

Analytical

- Limit of Quantitation below ng/L levels might be needed for marine systems.
- Improve method selectivity.
- Improve structure identification of environmental metabolites.
- Identify priority compounds for screening.
- Further develop methods for novel compounds and lower concentrations.
- Joint lab collaboration of sampling and method development and spectra databases.

Occurrence, Processes and Behavior

- Identify major sources of contaminants for coastal areas.
- Surveys in selected coastal areas (for target compounds).
- Special attention to estuaries, deltas, lagoons, and coastal outfalls.
- Start exploring if similarly acting compounds with similar structure can be grouped.

Biological Effects

- Assess sublethal, chronic effects.
- Assess biological uptake (invertebrates, phytoplankton, fish).
- Include dietary sources in addition to dissolved exposures.
- Evaluate effects of environmentally realistic concentrations (down to ng/L concentrations).
- Start to assess mixture toxicity.

Risk Assessment

- Establish databases with realistic data on: sources, behaviour, exposure and effects.
- Start exploring if similarly acting compounds with similar structure can be grouped.

Pathogens

- Improve risk assessment.
- Consider pathogenic microorganisms in an ecological context.
- Establish early warning systems.
- Improve data base on inputs, concentrations, effects in coastal Mediterranean.
- Combine sampling efforts for pathogens and pollutants.

Novel contaminants in marine settings: incremental science or a challenge for environmental studies

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INTRODUCTION

In 1983 Stumm *et al*. published their article "*From environmental analytical chemistry to ecotoxicology – A plea for more concepts and less monitoring and testing*". Since that time Schwarzenbach *et al*. (one of the co-author of the above cited article) published "*Environmental Organic Chemistry*" (1994 and 2003 second edition) providing in their book essential physical and chemical laws and principles govering the behavior and biogeochemical fates of organic compounds in natural systems. In the last two decades evidence has mounted on the global spread of certain persistent organic compounds (such as many organochlorine compounds); this has been accompanied by an increased quantitative understanding of the mechanisms influencing their distribution in diverse environmental reservoirs, including biota. There have also been many studies on the biochemical reactivities and potential for toxic effects (e.g., immunotoxicity, endocrine disruption, carcinogenicity) of various organic pollutants in animals, including humans. Recently, the endocrine disrupting effects of some organic contaminants have been recognized as having major impacts on wildlife (Esher and Hermens, 2002).

New and improved analytical techniques have led to the identification and quantification of many, previously undetected, organic anthropogenic compounds in the environment in recent years. These compounds include polybrominated diphenyl ethers – PBDEs (Sellström and Jansson, 1995; de Boer *et al*., 1998); perfluorochemicals: perflurooctane sulfonates - PFOS and perfluorooctanoic acid – PFOA (Giesy *et al*., 2001; Kannan *et al*., 2001); compounds – nonyl- and octylphenol; many pesticides such as triazine and phenyl ureas herbicides (Thurman *et al*., 1992; Buser, 1990; Tronczynski *et al*., 1993); veterinary and human pharmaceuticals; biocides and bactericides such as TBT and triclosane (Halling-Sørensen *et al*., 1997; Kolpin *et al*.,. 2002; Singer *et al*.,. 2002); phthalate esters (Mackintosh *et al*., 2004), and others. Moreover, it has been surmised that the presence of certain of these "novel" contaminants might indeed be of environmental concern, because they have been shown to be mobile, persistent and toxic and some are bioaccumulative. Some studies have also shown that the levels of certain of these chemicals increased over time and that their presence in the environment is widespread (e.g., PBDEs, Ikonomou *et al*., 2002; Johansson, 2004).

These findings and the identification of more and more toxic chemicals in the environment raise a question of how should we, as environmental chemists and scientists, approach the problem? Perhaps, it is also a question of how to avoid an incremental science, by only reporting the identification of "novel" man-made chemicals in the environment. Certainly it is a question of

how to reveal the significance of these findings. For environmental scientists, the identification of "novel" organic compounds whose presence could be of environmental concern reinforces a need for a rational and systematic approach to assess the environmental fates and effects of these chemicals. This includes a need for well-designed and well-balanced research that would include experimental, field and modelling studies. However, to clearly reveal the significance of the work and to validate assertions of models and experiments, a reliable set of baseline data is now essential. Further, appropriate monitoring strategies should allow better interpretation of temporal and spatial trends in contaminant concentrations determined with long-term observations. The CIESM Workshop on Novel Contaminants and Pathogens in coastal waters should help in certain aspects of this effort.

The question we want to raise and discuss is: what are appropriate strategies for studies and monitoring of the chemical contamination in the marine environment? For this discussion we bring two examples of the results of our studies concerning:

- Contamination of coastal waters by "novel" antifouling agents;
- Temporal trends of polybrominated diphenyl ethers as an example of "novel" contaminants in the marine environment.

We believe these examples illustrate the need for coordinated research and monitoring efforts.

ASSESSMENT OF THE EXTENT OF CONTAMINATION OF THE FRENCH COASTLINES BY ANTIFOULING AGENTS ("BIOCIDES BOOSTER")

National and international legislation has been introduced restricting the use of organotin biocides in antifouling paints for marine vessels. A number of replacement biocides are being used and, although generally based on copper metal oxides, also include organic antifoulants ("booster biocides") to enhance the coatings efficacy (Thomas, 1998). The contamination of coastal waters by novel antifouling agents is an example of chemicals of concern in the marine environment. However, detailed information on the levels, spatial and temporal variation of concentrations, and fates of these compounds are rather slim. The ACE (Assessment of Antifouling Agents in Coastal Environments: http://www.pml.ac.uk/ace/default.htm) project has completed environmental studies in European coastal waters of the following booster biocides: Irgarol® 1051 (2-methylthio-4-tertiary-butylamino-6-cyclopropylamino-s-triazine); dichlofluanid (N'–dimethyl-N-phenyl sulphamide); chlorothalonil (2,4,5,6-tetrachloro iso phthalonitrile); SeaNine® 211/Kathon 5287 (4,5-dichloro-2-n-octyl-4-isothiazolin-3-one); and diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea).

The study of the extent of contamination of the French coastlines by biocides booster compounds relied on sampling at 42 stations from the oceanographic vessel OV "Europe" during summer 2000 along the French Mediterranean coast between Monaco and Marseille, including Corsica, including a few marinas and harbors on these coasts. Some results of this study are presented below (Tronczynski *et al*., 2001). The Mediterranean coast represents the highest density of pleasure craft installations in France. Samples were collected in marinas with high pleasure boat activity and in open coastal waters in order to look for dissipation of chemicals outside of the harbors. Sampling stations are shown in Figure 1. Antifouling agents were extracted from filtered water samples by SPE and quantified by GC/NPD and GC/MS.

The compounds identified in the waters samples were chlorothalonil, irgarol, and its major degradation product (2-methylthio-4-tert-butylamino-s-triazine). Dichlofluanid was not detected in any samples.

Measurable concentrations of irgarol were detected in all samples except three samples collected off Corsica. These 3 sampling sites are representative of highly frequented open mooring areas during the summer period but not in direct connection to marinas. Concentrations ranged from 46 to 244 ng l^{-1} inside marinas and from 0.2 to 2 ng l^{-1} in coastal waters (Figure 2). Maximum concentrations are lower than those previously recorded in the area (Readman *et al*., 1993). The highest concentrations of irgarol were found in marinas with the highest pleasure boat activity, although no statistically significant relationship was found. The relationship would certainly be

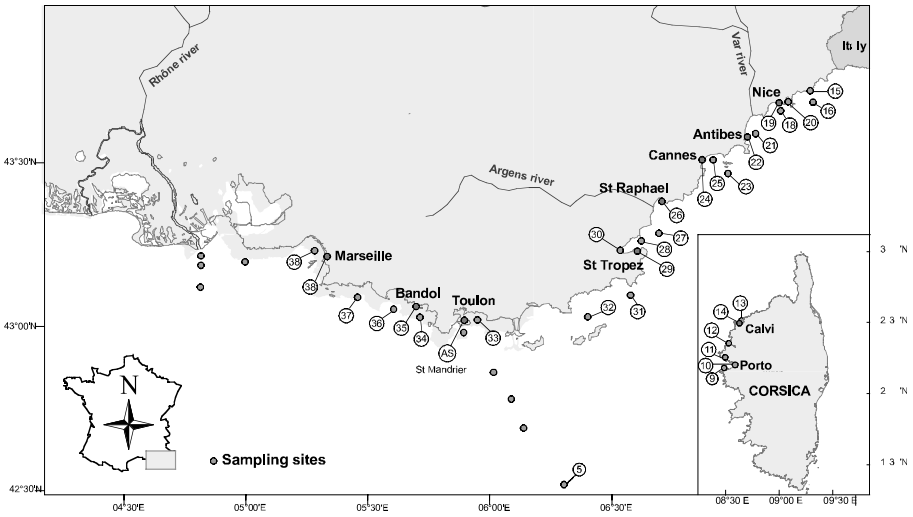

Fig. 1. Location of sampling stations along the French Mediterranean coasts, August-September 2000 cruise on oceanographic vessel "N/O L'Europe". Red dots are for samples collected inside marinas. AS is for the sample site studied during the annual survey.

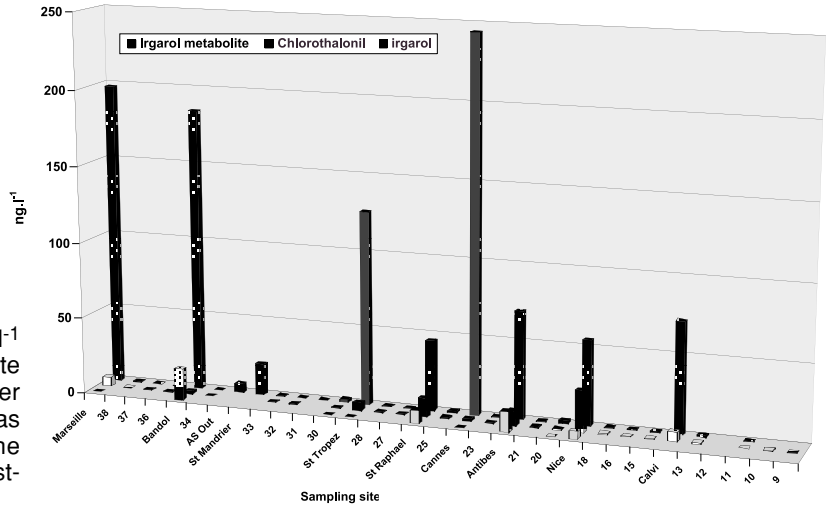

Fig. 2. Concentrations in ng l^{-1} of irgarol, irgarol metabolite and chlorothalonil in water samples collected in marinas and coastal areas of the French Mediterranean coastline, August 2000.

better explained if the number of boats instead of the number of berths available in marinas would have been taken into account.

The major degradation product of irgarol (GS26575 or M1) was identified only in some marinas at concentrations between 5 and 21 ng l^{-1}. The highest concentrations of irgarol were not necessarily associated with detectable levels of the metabolite. Concentrations of chlorothalonil were between undetectable levels (< 0.05 ng l^{-1}) and 27 ng l^{-1}, with higher levels recorded inside marinas (1.7 to 27 ng l^{-1}) compared to coastal waters (0.1 to 0.9 ng l^{-1}). The higher concentrations of chlorothalonil were not associated with higher concentrations of irgarol.

Among the 3 antifouling agents studied irgarol show the highest concentrations in all water samples, although dichlofluanid and chlorothalonil were found to be sold in more important quantity in paints. These results are related to the higher water solubility and higher persistence of irgarol, and demonstrate the higher dissipation capacity of this compound into coastal waters.

LEVELS AND TRENDS OF POLYBROMINATED DIPHENYL ETHERS (PBDES) AND OTHER ORGANOHALOGEN COMPOUNDS IN ARCHIVED MUSSEL SAMPLES 1981 – 2003

Polybrominated diphenyl ethers (PBDEs) have been used as chemical flame retardants since the 1970s. The production and use (in many plastics, textiles, polyurethane foams and electronic

circuits) of these chemicals has increased exponentially since 1980. In parallel to this increase, their environmental concentrations have risen very rapidly in biological and sediment samples from different marine regions (Ikonomou *et al*., 2002; de Boer *et al*., 2003; Law *et al*., 2003; Johansson *et al*., 2004). On the other hand, a few recent reports have shown a decline in the last few years of PBDE levels in the environment and human milk and suggested that this reduction may reflect regulatory measures taken in Europe (Kirkegaard *et al*., 2004; Sellström *et al*., 2003; Joahansson *et al*., 2004). These trends should also be compared with decreasing levels of better-studied organohalogen compounds such as polychlorinated biphenyls (PCBs). The long term environmental monitoring enables temporal trends to be discerned of concentration levels in the marine environment. There are different ways of performing observation programs but marine mussels, as filtering organisms, have proven to be useful for biomonitoring and assessment of coastal contamination at a particular site (Phillips, 1980). In our study, we have undertaken the retrospective analysis of "classic" and "novel" persistent organic contaminants in archived samples of marine mussels (*Mytilus edulis*) collected over the past 22 years by the French National "Mussel Watch" Network (RNO). The aim of this study was to achieve a better understanding about levels and trends for PCBs and brominated diphenyl ethers (BDEs) (Johansson *et al*., 2004). Indeed, the comparison between the trends of already banned and novel organohalogen compounds raises new research questions.

In this example the samples analyzed were samples collected at Villerville in the Seine Estuary during the period 1981-2003. The mussel samples selected for the chemical analysis were collected each year during the same period so that any differences in contaminant concentrations could not be related to spawning or differences in the physiological states of the mussels. Living mussels were depurated, shucked, homogenized and stored at –20°C before freeze-drying. Each archived sample consisted of a pooled sample of about 20 mussels of homogeneous size. Detailed description of the analytical procedure is given in Johansson *et al*. (2004).

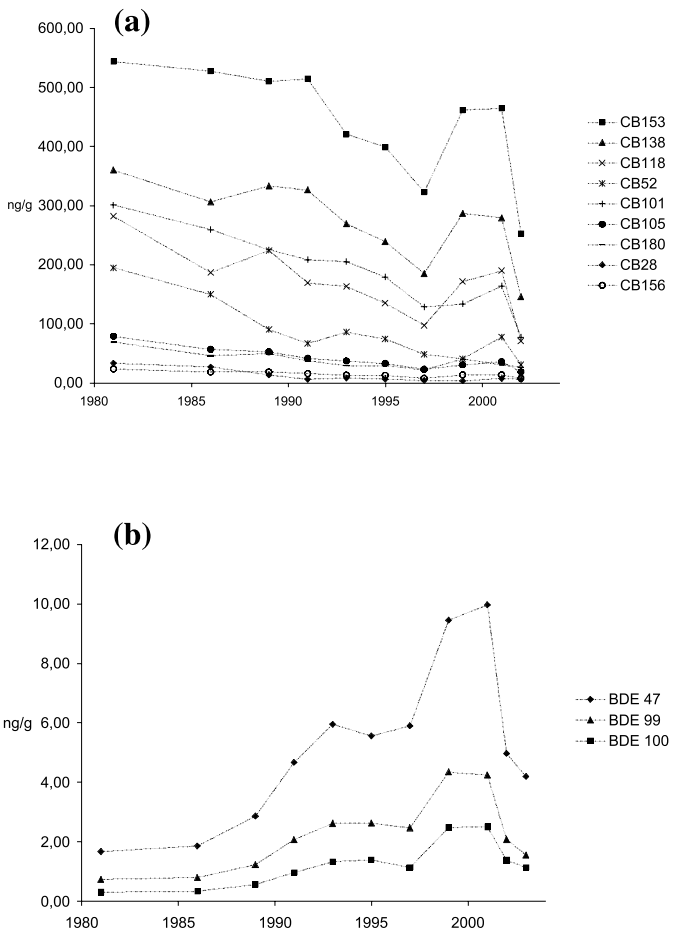

Fig. 3. Time trends of selected CBs (a) and BDEs (b) in mussels from the Seine Estuary 1981 – 2003; concentrations in ng g^{-1} dry weight.

The overall trend for the levels of selected chlorobiphenyls (CBs) and most prevalent congeners of PBDEs (BDE-47 > BDE-99 > BDE-100) are shown in Figure 3. A significant decrease of concentrations of individual CBs is noted between 1981 and 1997. During 1999 and 2001 a notable increase was observed in the levels of the dominant CB-congeners and the concentrations increased to the same levels as in the early 1990s. In 2002 the levels had dropped to the lowest levels.

Conversely, the concentrations of the three most prevalent congeners BDE-47, BDE-99, and BDE-100 increased greatly in mussels from the Seine estuary between 1981 and 2001. The rate of increase was almost the same for BDE-47 and BDE-99. Highest concentrations were found in the samples from 1999 and 2001.

Increasing concentrations of PBDEs during the 1980s have also been observed in other retrospective temporal trend studies. A study of guillemot eggs from the Baltic Sea showed that the concentrations of PBDEs increased from the beginning of the 1970s, but from the mid-1980s they decreased rapidly (Sellström *et al*., 2003). A similar trend was also found in a study of PBDEs in pike from Swedish waters, however, the decreasing trend was considerably slower (Kirkegaard *et al*., 2004). A decrease of levels has also been observed for BDE-47 and BDE–99 in eel, cod liver and sediment from Dutch rivers, the southern North Sea, and Ireland from 1995 to 2001 (de Boer *et al*., 2003). In ringed seals from the Canadian Arctic the levels of PBDEs have been reported to increase exponentially since the beginning of the 1980s, and until 2000, no decline of the levels was observed (Ikonomou *et al*., 2002).

These results indicate that temporal trends of PBDE levels in various environmental samples show some regional differences. However, the observed decline of the PBDEs in environmental samples is not yet fully understood. This decline could be related to the decrease in the use of the commercial penta-BDE mixture in Europe (Renner, 2000). Furthermore, in comparison with classic organochlorine compounds such PCBs, declining levels of PBDEs in environmental samples occurs fairly rapidly. In the mussels of the Seine estuary, the PBDE concentrations started to decline relatively late (only since 2002) in comparison to some other sites in Europe. We also found that the highest concentrations of PBDEs, in samples from 1999 and 2001, coincided with unexpected increases of PCB concentrations in these samples. This increase in organohalogen concentrations in the mussels at this estuarine station could be related to large floods of the Seine River during these years. The large loads of suspended matter during floods, as well as a flushing out of the deposited sediments from the internal estuary of the Seine during this period, would have discharged significant amounts of sediment-associated organohalogens. Furthermore, recent data reveal pulses of banned POPs and increases in their concentrations in North American air samples (Buehler, 2002). These results indicate that release of the banned organohalogen compounds can continue from different sources. It appears also that these compounds are now embedded in natural biogeochemical cycles and that natural events such river floods can mobilize these compounds, potentially in a bioavailable form.

New contaminants in the North Sea and Baltic Sea

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Starting point for the investigation of novel contaminants in the marine environment are the lists of Priority Pollutants generated by various international organisations, such as the EU (EU-Water Framework Directive: EU-WFR), OSPAR Commission (for the protection of the Marine Environment of the North-East Atlantic) and HELCOM Commission (Baltic Marine Environment Protection Commission).

Some of the compounds in these lists have been monitored for many years (e.g. PAH and chlorinated hydrocarbons (CH)); nearly all of them are lipophilic and can be analysed by GC-MS techniques on a routine basis.

In contrast to this, most of the «newer» hazardous substances are more polar and/or need special analytical procedures - thus knowledge on these substances in the marine environment is limited or even non-existent.

Polar Pesticides

Most modern pesticides are more polar than the classical ones and often incompatible with direct GC techniques; therefore, a HPLC-MS technique was chosen as the general analytical approach. Because of the expected low concentrations in marine samples a tandem MS procedure with multi reaction monitoring (MRM) is necessary. A solid phase extraction (SPM) at HRP resin is used as extraction and enrichment technique.

As the river Elbe is the most important source for contaminants in the German Bight, sampling in its estuary allows a good overview of possible pollutants entering the North Sea. Table 1 summarises typical concentrations observed at the mouth of the river Elbe. Out of 37 pesticides analysed, 21 were detected. The most abundant compounds are Diuron, Isoptoturon, Atrazin, Irgarol, Terbuthylazine, Mecoprop, and 2,4-D. Not all of the pesticides under investigation (e.g. some phosphorous ester insecticides) are appropriate for ESI-MS-MS techniques; therefore, about 10 compounds are now analysed by GC-NCI-MS. By this method, Chlorpyrifos-ethyl, Trifluralin and Endosulfan are detected in the Elbe as well.

The geographical distribution of the most important triazine and phenylurea herbicides in the North is shown in Figures 1 and 2. For all compounds there is observed a distinct gradient from the coasts to the open North Sea. Besides the Elbe, the rivers Schelde and Rhine are distinct sources. The elevated concentrations at the Norwegian coast are primarily due to the water outflow from the Baltic Sea. In Table 2 the concentrations are summarised and grouped into 5 classes; here it can be seen that only 5 compounds can be determined (with the present LODs) in the open sea; these are Diuron, Fenuron and Isoproturon, Atrazine and Terbuthylazine.

Examples for the distribution in the western Baltic Sea are shown in Figure 3 for Diuron and Isoproturon. While they show quite uniform distributions in the open sea, they show distinct differences in coastal areas. The high values of Diuron at the western stations, paralleled by high concentrations of Irgarol, are probably due to shipping activities in those areas; both compounds are used in antifouling paints. The distribution of Isoproturon - and similar those for 2,4-D, Terbutylazin and Atrazine - shows that the river Odra is an important source for these herbicides. Simazine exhibits a third distribution pattern with quite even, but relatively high concentrations ranging from 1.5 to 4.5 ng/L. This structure can be explained best by atmospheric inputs and/or old burdens.

The time series (starting in the year 2000) are too short for the investigation of temporal trends. The investigation of seasonal effects in the river Elbe (Figure 4) shows that there are large concentration differences for some compounds during the year, which can be explained by the application periods for the different pesticides. The significance of such seasonal effects in the marine area is yet unknown. However, for other pesticides, e.g. Lindane, seasonal effects have been observed in the North and Baltic Sea in the past as well.

In Figure 5 the distribution of some additional compounds from the EU-WFD list is shown. Trifluralin, Chlorpyriphos and Endosulfan can be detected in the southern part of the North Sea at fairly low concentrations (0.01 to 0.1 ng/L). These compounds can be analysed very sensitively by GC-NCI-MS.

Polyfluorinated organic acids, which have been shown to be very persistent in the environment, are a group of large volume chemicals with many applications. They can be analysed by negative ESI HPLC-MS-MS with a good sensitivity. A chromatogram example for a sample from the German Bight is shown in Figure 6. First results from a survey in the North Sea in Aug. 2003 are presented in Figure 7. Again the river Elbe is a source for these compounds. Highest values are observed for PFOA and PFOS. (The absolute values have to be considered with some care, as validation of the method has not yet finalised).

Tributyl tin (TBT) is one of the most toxic compounds in the marine environment and has biological effect levels of about 1 ng/L. As Figure 8 shows, these levels are observed in sea water of coastal areas of the German Bight. The toxicity level is exceeded in the river Elbe or the Kiel canal. Analysis is done after derivatisation with $NaB(Et)_4$ by GC-AED (or GC-MS).

In addition to the analysis of target compounds, a non-target analysis on selected samples is also performed to determine new compounds in the sample extracts. This is felt to be necessary in order to avoid “blind” spots because of the very specific analytical procedures generally used. These screening investigations will be complemented by toxicological investigations. Some newer results will be presented.

Table 1. Pesticide concentrations [ng/L] in the river Elbe.

	Stade May 2003		Stade May 2003		Stade May 2003
Alachlor	<0.5			2,4-D	2,3
Metamitron	<5	Azinphos-ethyl	<0.2	Clofibrinsäure	8,3
Metazachlor	4,3	Azinphos-methyl	<0.3	Dichlorprop	5,2
Metolachlor	1,9	Chlorfenvinphos	<0.2	MCPA	5,4
Pendimethalin	<1	Chlorpyriphos-ethyl	<1	Mecoprop	7,8
Chlortoluron	4,5	Chlorpyriphos-methyl	<2.5	Atrazin	15,6
Diuron	60,0	Diazinon	<0.3	Irgarol	10,5
Fenuron	5,0	Dichlorvos	<10	Metribuzin	<2
Isoproturon	30,7	Dimethoat	2,0	Prometryn	5,3
Linuron	1,2	Fenitrothion	<25	Propazin	0,8
Monolinuron	0,7	Fenthion	<1	Sebuthylazin	<0.3
		Malathion	<0.3	Simazin	4,0
		Parathion-ethyl	<4.5	Terbuthylazin	7,2
				Terbutryn	3,1

Table 2. Average concentrations [ng/L] in 2003 in various regions of the North Sea.

	Average River (Elbe)	Average Coastal	Average Intermediate	Average Open North Sea	Average Norwegean Coast
Metazachlor	2,44	0,73	0,25	<	0,22
Metolachlor	6,49	1,24	0,34	<	<
Chlortoluron	6,15	3,34	0,98	<	0,46
Diuron	57,17	15,26	3,39	0,39	3,85
Fenuron	3,90	2,48	0,97	0,41	1,04
Isoproturon	10,81	4,04	1,42	0,10	0,81
Atrazin	39,35	4,49	2,51	0,71	2,57
Irgarol	5,32	0,69	0,14	<	1,02
Prometryn	4,19	0,67	<	<	<
Simazin	6,49	1,33	0,70	<	0,87
Terbuthylazin	13,96	2,27	1,02	0,31	1,11
Terbutryn	4,81	1,14	0,35	<	0,26
Clofibrinsäure	3,38	0,75	0,53	<	<
MCPA	2,17	0,52	0,62	<	0,35
2,4-D	2,50	0,52	0,48	<	0,37
Dichlorprop	2,07	0,65	0,69	<	<
Mecoprop	3,36	1,08	0,75	<	0,37

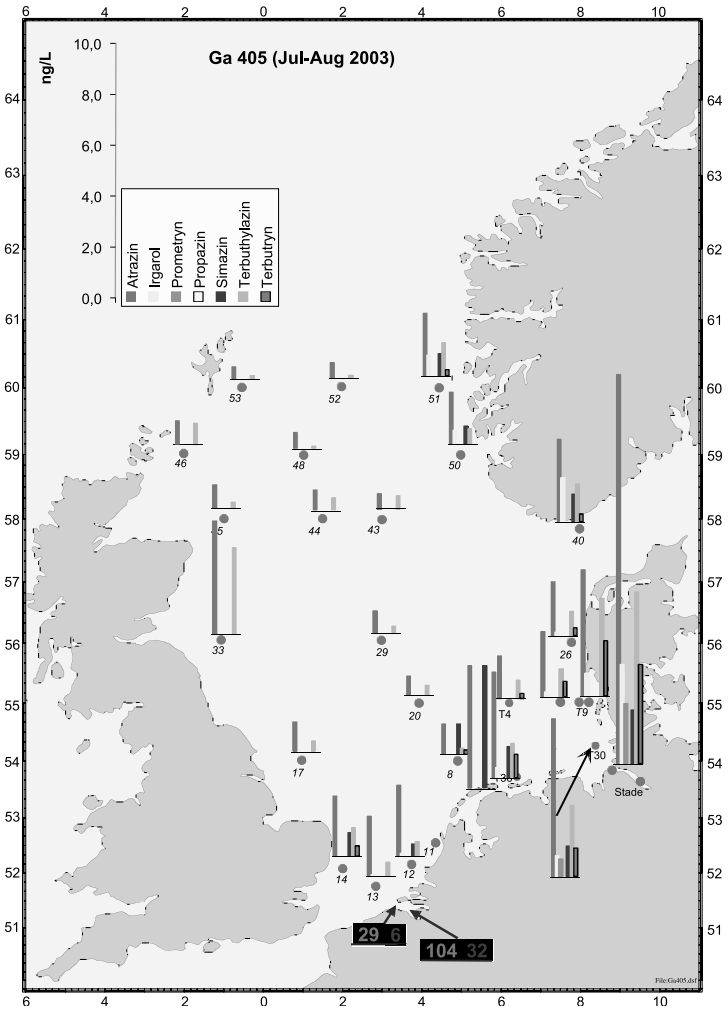

Fig. 1.
Distribution of Triazine herbicides in surface water (5m) in Aug. 2003 [ng/L].

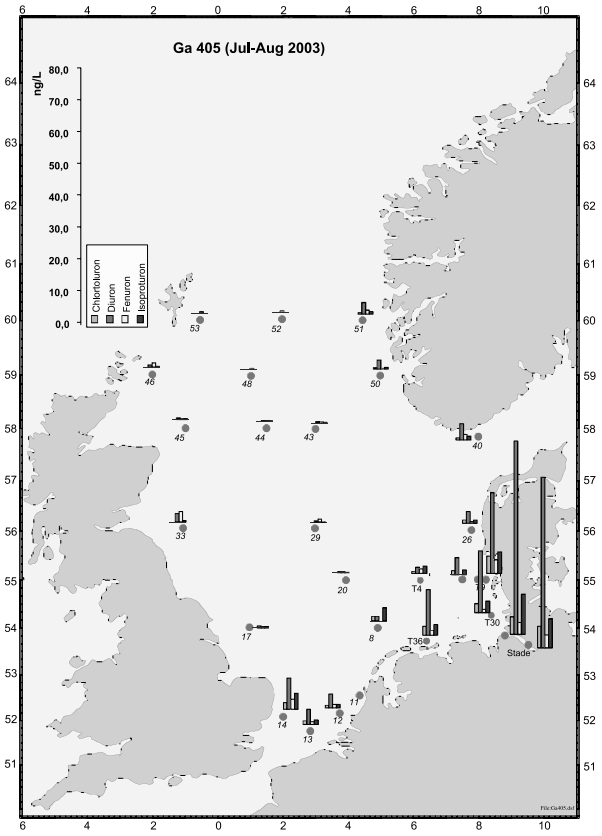

Fig. 2.
Distribution of Phenylurea herbicides in surface water (5m) in Aug. 2003 [ng/L].

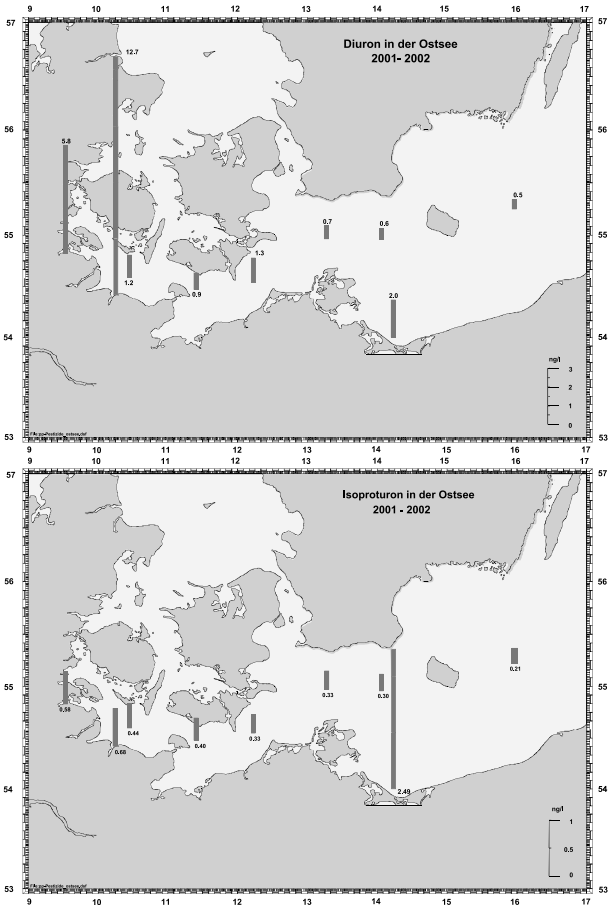

Fig. 3.
Distribution of Diuron and Isoproturon in surface water (5m) of the western Baltic Sea [ng/L].

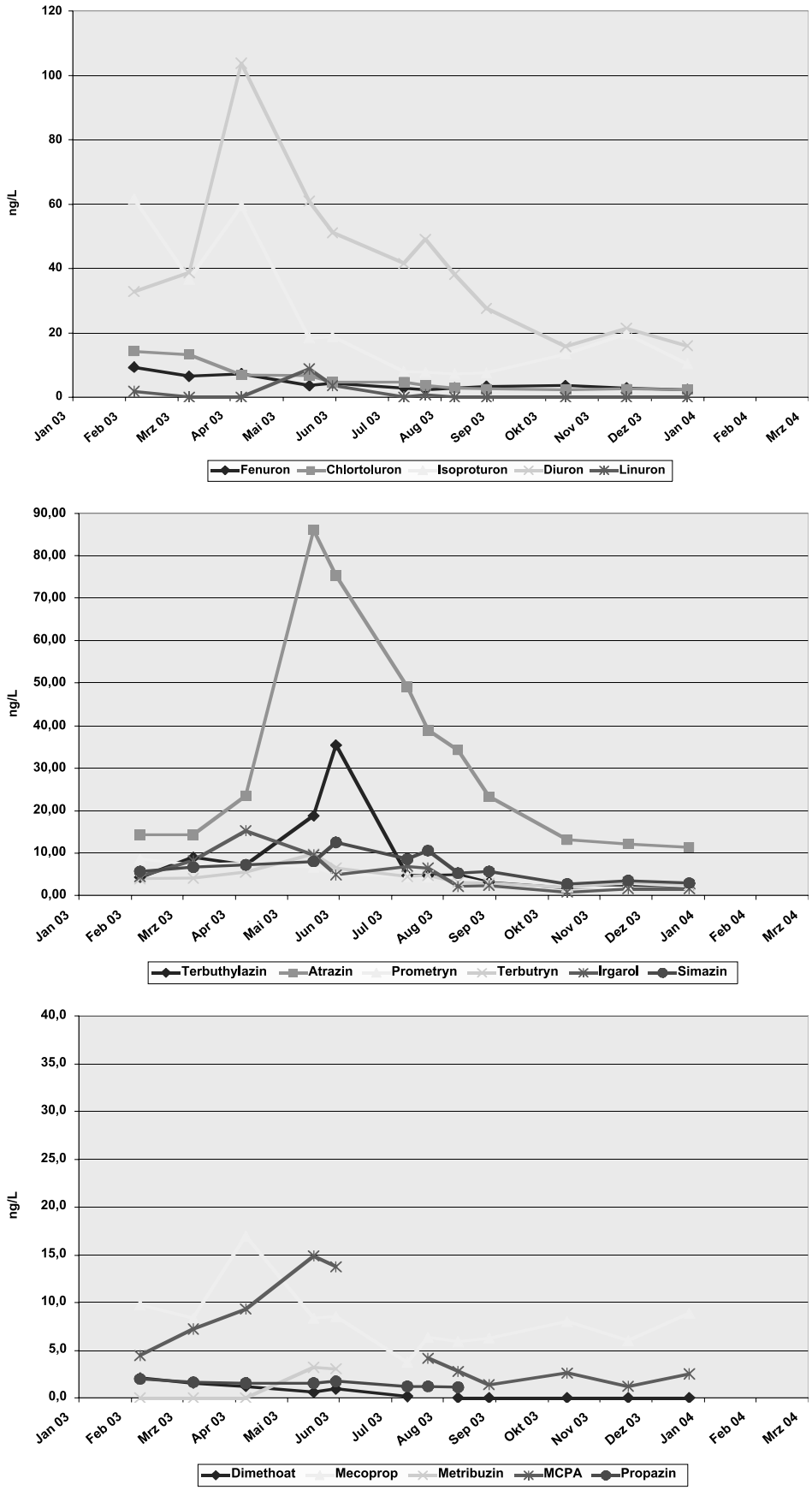

Fig. 4. Concentrations [ng/L] of various pesticides in the river Elbe during the year 2003.

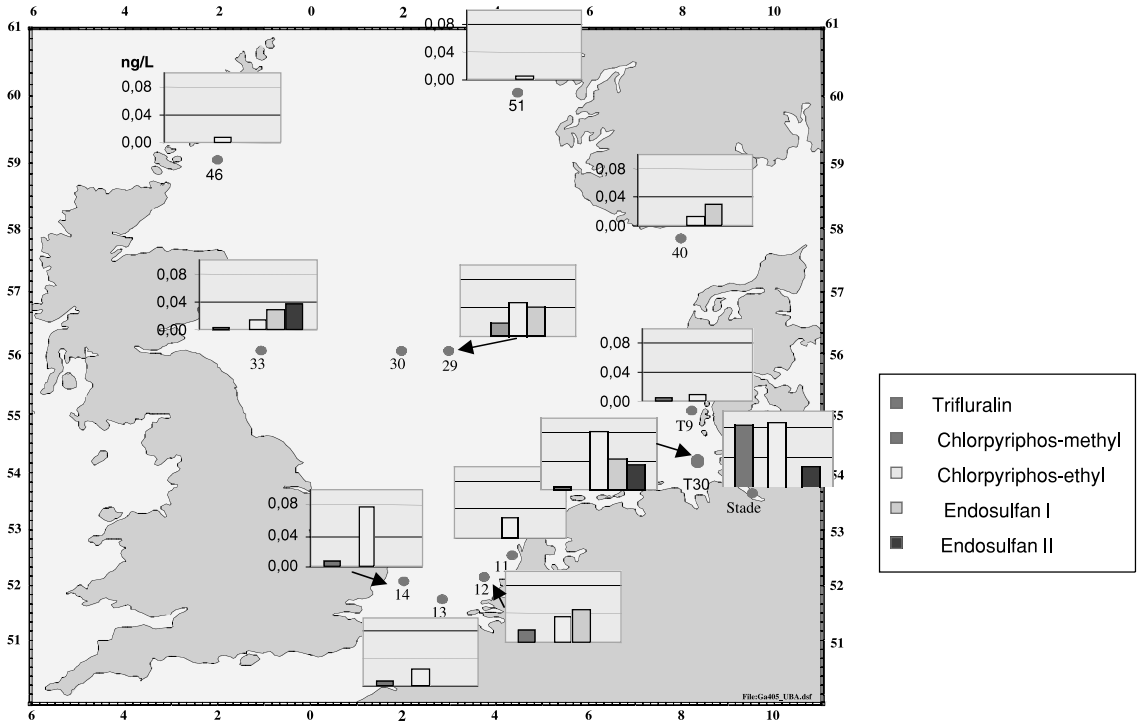

Fig. 5. Distribution of selected pesticides in surface water (5m) in Aug. 2003 [ng/L].

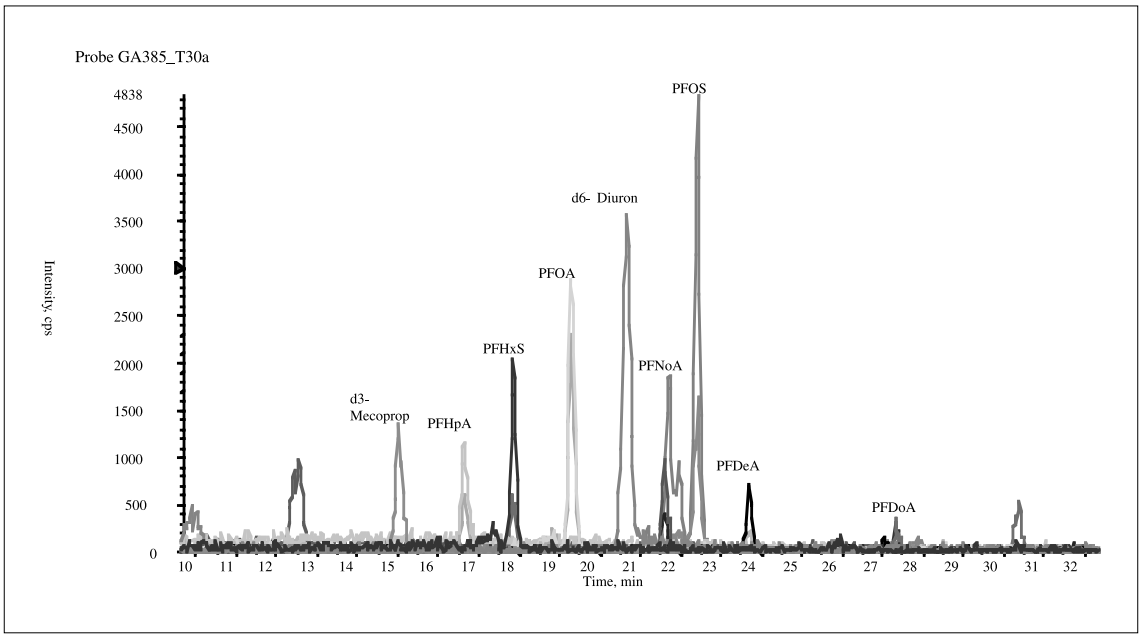

Fig. 6. HCPLC-MS-MS chromatogram (MRM traces) of polyfluorinated organic compounds of a water sample from the German Bight.

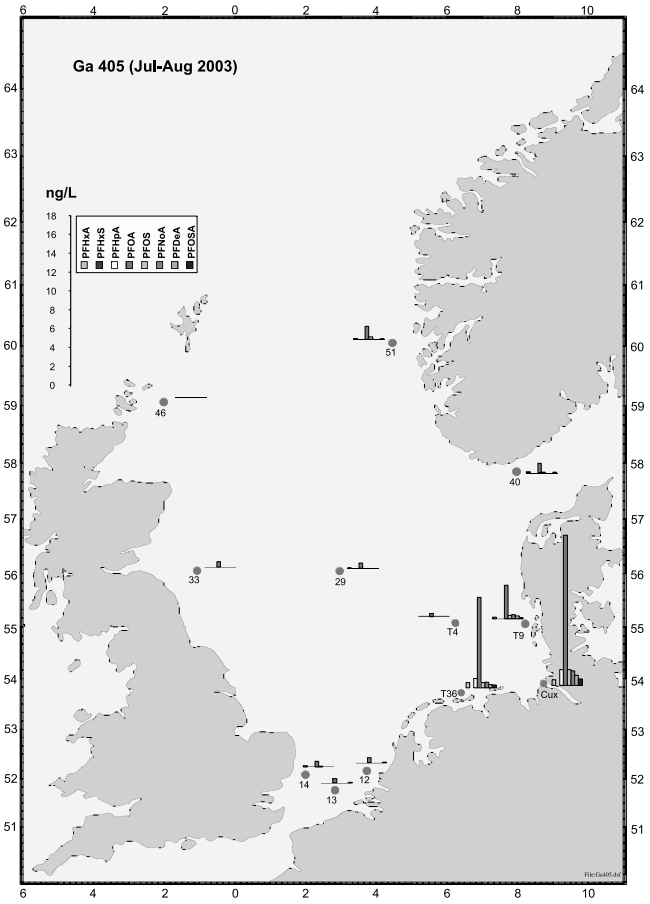

Fig. 7. Distribution of polyfluorinated organic compounds in surface water (5m) in Aug. 2003 [ng/L].

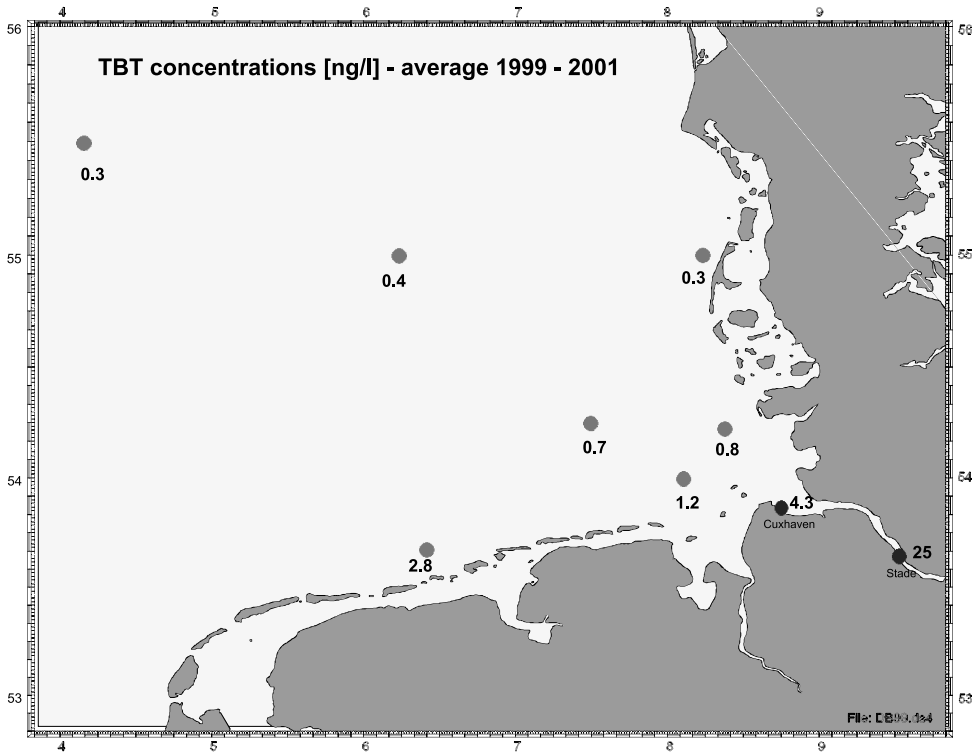

Fig. 8. Distribution of TBT in surface water (5m) [ng/L].

Input and behaviour of alkylphenolic endocrine-disrupting contaminants in a stratified estuary and coastal waters

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INTRODUCTION

Aromatic surfactants of alkylphenol polyethoxylate (APnEO) type with estimated annual worldwide production rate of about 6 x 10^5 tons belong to the most popular surfactant classes. Due to their widespread use in such high volumes and considerable toxicities to aquatic life, APnEO should be considered potentially important environmental contaminants (Servos, 1999; Ying *et al*., 2002).

It was shown in model laboratory experiments as well as in sewage treatment plants and various types of ambient waters that APnEO exhibit a complex metabolic behaviour, yielding a variety of relatively stable metabolic products. Biotransformation of APnEO is very complex and usually starts at the hydrophylic part of the molecule, resulting in a rather unique metabolic pathway, which is characterised by the formation of various stable intermediates (Ahel *et al*., 1994) (Figure 1). Some of these metabolites are more lipophylic and therefore more toxic to aquatic life than the parent molecules (see review by Servos, 1999). In the last decade, the interest in ecotoxicological effects of these lipophylic metabolites has dramatically increased because of their proven endocrine-disrupting potential (Jobling and Sumpter, 1993; Jobling *et al*., 1996; Metcalfe *et al*., 2001; Johnson and Sumpter, 2003). The most recent studies have shown that APnEO intermediates with shorter EO-chains are further transformed by microbial carboxylation of highly branched alkyl-chain, yielding a suite of more polar metabolites, which also exhibit a significant persistence (Ding *et al*., 1996; Di Corcia *et al*., 1998). However, these metabolic products are less toxic than the metabolites containing alkylphenol moiety, while their potential for endocrine disruption seems to be insignificant (Johnson and Sumpter, 2003).

Owing to their hydrophobic moiety, APnEO show a significant affinity for suspended particles, which results in a widespread presence of surfactant residues in aquatic sediments and biota (Bennie, 1999). The adsorption onto sediment and bioaccumulation are particularly pronounced for the lipophylic degradation products of APnEO such as nonylphenol (NP), nonylphenol monoethoxylate (NP1EO), and nonylphenol diethoxylate (NP2EO).

Coastal and estuarine waters receive large quantities of aromatic surfactants, either directly from municipal wastewaters or indirectly from polluted rivers. In the past decade, there have been an increasing number of papers dealing with the behaviour and fate of APnEO in coastal and estuarine environments (Marcomini *et al*., 1990; Blackburn *et al*., 1999; Isobe *et al*., 2001; Ferguson *et al*., 2003; Jonkers *et al*., 2003). In estuaries, most of the published data deal with well-mixed macrotidal estuaries, while the studies in stratified estuaries, characteristic of the Mediterranean Sea, are relatively scarce. There are two major motivations to study behaviour and

fate of surfactants in estuarine environments. Many of the world estuaries are densely populated and represent areas of intensive and often mutually conflicting anthropogenic activities including fishery, industry, navigation and tourism. On the other hand, estuaries are well known as important regions of biological diversity and as such, potentially highly vulnerable to environmental stress imposed by different human activities, including those characterised by extensive introduction of man-made chemicals.

This paper summarizes our studies (Kvestak and Ahel, 1994; Kvestak *et al*., 1994; Kvestak and Ahel, 1995) conducted in the estuary of the Krka River, Croatia, and discusses environmental behaviour and fate of APnEO and their metabolites in this unique system characterised by sharp salinity gradients. In addition, the results of the recent screening of endocrine-disrupting compounds, including surfactant-derived alkylphenolic compounds and bisphenol A, in Croatian coastal waters are also presented.

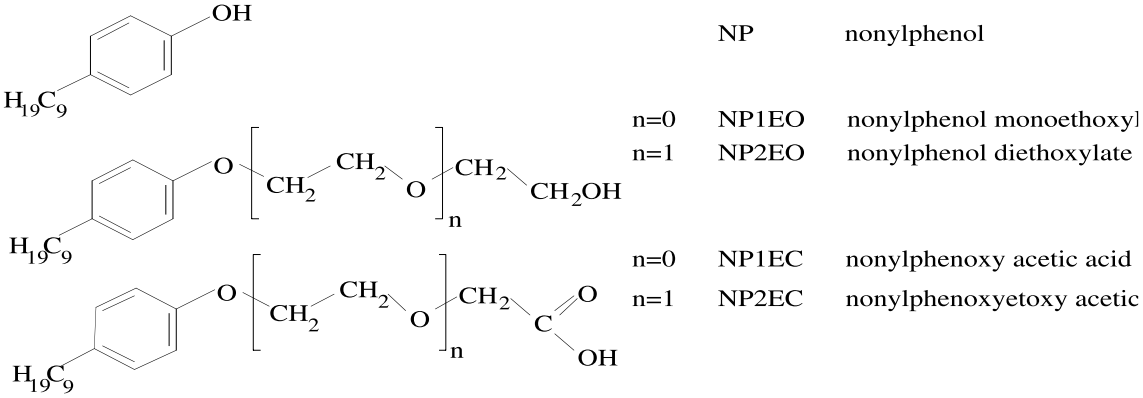

Fig. 1. Structures and acronyms of the most important estrogenic metabolites of nonylphenol polyethoxylates.

SITUATION AND HYDROGRAPHIC FEATURES OF THE PERMANENTLY STRATIFIED KRKA RIVER ESTUARY

The studies presented in this paper were carried out in the Krka River estuary situated in the middle part of the eastern Adriatic coast. The total length of the estuary is about 30 km and the depth varies from few meters, immediately below the waterfalls, to 40 meters at the sea-end of the estuary (Sibenik Harbour). Owing to its unique hydrographic features, the Krka River estuary belongs to the most intensively investigated estuaries of the Mediterranean Sea with a special emphasis on the processes that occur at estuarine boundaries. The Krka River estuary is a typical karstic estuary characterised by low terrigenous input of suspended materials and conspicuous vertical salinity gradients, resulting in a very stable stratification throughout the year (Figure 2). The upper freshwater and lower seawater layers are separated with a visible interface situated at the halocline, which contains an organic film formed mainly by accumulation of plankton-derived organic matter (Zutic and Legovic, 1987). The depth of the halocline fluctuates seasonally, depending on the river flow, between 0.2 and 6 m.

It was shown that such stratified estuaries are particularly suitable for studies of environmental behaviour of different constituents since the entire range of estuarine master variables can be achieved at one single station, which significantly simplifies the required sampling strategy. On the other hand, the Krka River estuary shows a conspicuous change of anthropogenic pressure along its longitudinal profile. The largest part of the estuary is a pristine environment situated within the borders of a National Park area with only few smaller settlements and very limited industrial activities, while the main source of anthropogenic input are wastewaters of the city of Sibenik (40000 inhabitants), which enter the estuary in its lower part, i.e. in the area of Sibenik Harbour. Municipal and industrial wastewaters are discharged into the estuary without any pretreatment through several outlets along the northern shoreline of the Sibenik Harbour basin.

Since 1989, several sampling campaigns have been performed, comprising sampling of wastewaters and estuarine water column. In order to determine spreading of wastewater plume

into the Sibenik Harbour, water samples were collected at different distances from the major sewage outlets at depths characteristic of brackish (0.5 m) and saline (6 m) layers. A more detailed sampling on the vertical profile of the water column, with a special emphasis on the brackish water-seawater interface, was performed at the station E4A, which is situated in the middle part of the Sibenik Harbour.

A nation-wide screening of alkylphenolic compounds was performed on several hot-spots along the eastern Adriatic coast as a pilot-study within the national monitoring programme (Project Jadran).

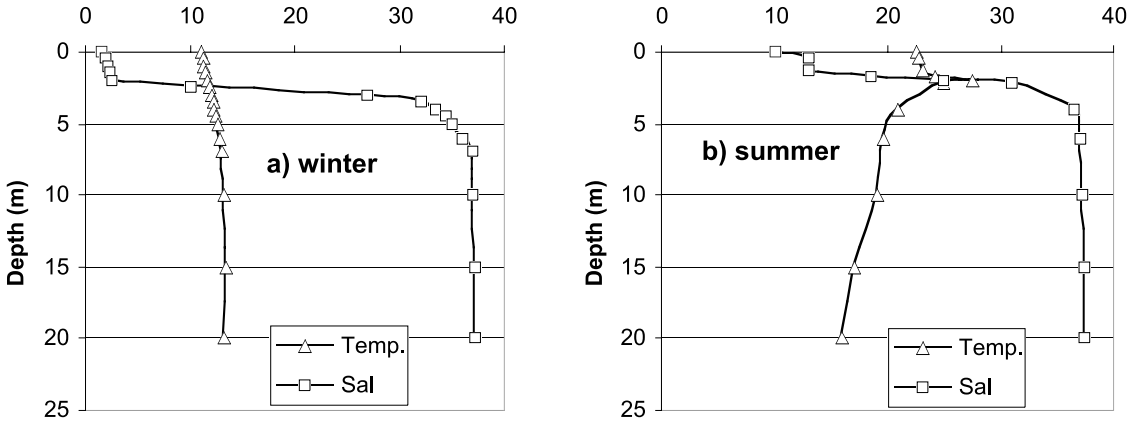

Fig. 2. Typical vertical profiles of temperature (°C) and salinity (PSU) in the water column of the Krka River estuary in winter and summer (Grzetic, 1990).

METHODS

NPnEO, including their stable metabolic products, were determined using an approach based on HPLC separation followed by spectrofluorimetric detection, which involved both reversed phase (separation of homologues and isomers) and normal phase (separation of oligomers) systems (Ahel and Giger, 1985a; Ahel and Giger, 1985b; Ahel *et al*., 2000). Liquid chromatography/tandem mass spectrometry (LC/MS/MS) was used for identification and confirmation purposes, as well for highly specific quantitative determination of alkylphenols and alkylphenol carboxylic acids (Petrovic *et al*., 2003). In order to investigate the partitioning behaviour of surfactant residues, water samples were previously filtered through glass fiber filters (GF/F) and the dissolved and particulate fractions were further separately processed. Briefly, dissolved fraction of both classes of aromatic surfactants was enriched from wastewater and estuarine waters using C_{18} solid-phase extraction, while the particulate fraction was extracted using an ultrasonically enhanced extraction. Lipophylic metabolites of NPnEO (NP, NP1EO and NP2EO) were determined by an alternative enrichment procedure, using a continuous steam distillation/solvent extraction in a special apparatus, followed by a direct analysis of the cyclohexane extracts by normal phase HPLC (Ahel and Giger, 1985a).

In addition to the determination of aromatic surfactant residues in the estuary, the die-away biodegradation experiments were performed to assess the behaviour and fate of the target compounds (Ahel and Kvestak, 1995). The experiments were performed using autochthonous microbial populations, which were sampled in different estuarine compartments as well as in different seasons. The biodegradation media were always kept at temperatures identical to those found in the ambient water at the moment of sampling.

CHARACTERISATION OF SURFACTANT INPUT

The input of APnEO surfactants into the Sibenik Harbour was determined by analysing wastewaters from all major outlets of the city of Sibenik (Kvestak *et al*., 1994). The homologue- and oligomer-compositions (Figure 3) found in wastewater extracts revealed the presence of common commercial mixtures of APnEO, which have been partially changed due to the physico-chemical partitioning and biotransformation in the sewer system. It should be stressed that a

significant percentage of APnEO (6-60 %) entering Sibenik Harbour via municipal wastewaters is bound to sewage particles. This fact markedly influences their fate in the estuary. The determination of aromatic nonionic surfactants in wastewater extracts by reversed-phase HPLC (Ahel and Giger, 1985b) indicated a strong predominance of nonyl homologues, while the presence of octyl homologues was insignificant (<5 %). The oligomer composition of NPnEO in the total wastewater samples displays a great deal of similarity to those commercial mixtures that are commonly used in detergent formulations, which are characterised by an oligomer distribution maximum at NP10EO.

The comparison of distribution patterns in the dissolved and particulate phases shows a pronounced selectivity in the partitioning of individual oligomers. The oligomer composition in the particulate phase is characterised by an enhanced abundance of lower oligomers, resulting in oligomer distributions that show typical maxima between NP6-9EO. This situation can be explained by decreased water solubilities and, consequently, increased lipophylicities (Ahel and Giger, 1993) of the lower oligomers. The observed change in the oligomer distribution patterns depends on the concentration of suspended solids in the wastewater sample. The typical maximum of oligomer distribution in the dissolved phase displays a shift of the maximum towards higher oligomers (NP11EO-NP12EO). It should be noted that the oligomer distributions presented in Figure 3 show only the distribution of higher oligomers (nEO>3), which are considered parent compounds (Ahel *et al.*, 1994). In addition to these parent oligomers, their lipophylic metabolites, including NP, NP1EO and NP2EO, were detected in all samples in lower but significant concentrations (typically below 10 μg/L of the individual metabolite). However, due to a rather short sewer system (residence time is estimated to be less than 1 hour), only a minor part of the parent compounds was biotransformed and the contribution of the lipophylic metabolites to the total concentration of nonylphenolic compounds did not exceed 5 %.

It is interesting to note that the concentration of aromatic surfactants varied significantly during the period covered by these investigations (1989-2003). In the late eighties, the average levels of the NPnEO were rather high (approximately 0.5 mg/L), suggesting an extensive usage of NPnEO in that period. Assuming an average discharge of municipal wastewaters of about 0.3 m^3/s, the input of NPnEO was estimated at 5 tons per year. As a consequence of risk reduction measures, which were introduced in Western Europe, as well as reduced industrial activities during and after the war in Croatia (1991-1995), the concentration of NPnEO decreased below 0.1 mg/L. However, they still represent ubiquitous contaminants in Croatian wastewaters.

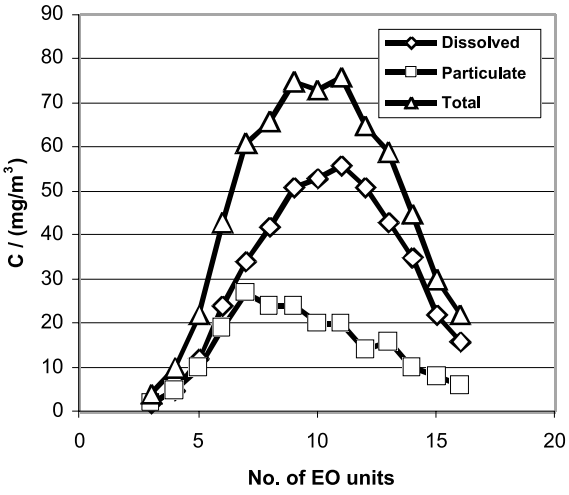

Fig. 3.
Distribution of oligomers of nonylphenol polyethoxylates (NPnEO) in dissolved and particulate fractions of municipal wastewaters Sibenik Harbour.

DISTRIBUTION IN THE WASTEWATER PLUME

The spreading of the wastewater plume in the Sibenik Harbour was investigated by taking the samples at different distances from the main outlet at two different depths characteristic of the brackish compartment (0.5 m; salinities <20 PSU) and marine compartment (6 m; salinities >37 PSU) of the estuary. The results indicate that spreading of wastewaters occurs almost exclusively

in the brackish layer, while the underlying saline layer remains hardly affected. Figure 4 shows the distribution of NPnEO in the wastewater plume along with the most important group of anionic surfactants, linear alkylbenzene sulphonates (LAS). As can be seen, there is a strong reduction (down to 1-10 μg/L) of the surfactant concentrations in the closest proximity (25 m) of the outlet. After a distance of only 100 m the concentration of both NPnEO and LAS in the plume dropped down below 2 μg/L. The observed effect depends strongly on wind conditions, which can enhance the velocity and direction of surface currents as well as mixing of the brackish and saline layers (Legovic *et al*., 1991). Such a fast reduction of surfactant concentration with increasing distance from the outlets can mainly be assigned to a very fast dilution; however, differences in the distribution patterns of individual homologues and/or oligomers suggest the importance of some other mechanisms that lead to the elimination of parent compounds. If the relative contribution of lipophylic metabolites in the total nonylphenolic compounds is plotted versus distance from the outlet (Figure 5), one can see that their percentage in the plume significantly increases (up to 54 %). This indicates possible importance of some oligomer-specific elimination process in the plume, most probably biotransformation of the parent NPnEO. Alternative mechanism, i.e. fast sedimentation of surfactant molecules with sewage-derived particles, is less likely since this process would tend to decrease the relative percentage of lipophylic metabolites. However, Marcomini *et al*. (1990) have shown that the association of the lipophylic metabolites with suspended particles is an important process leading to significant concentrations in the uppermost sediment layer.

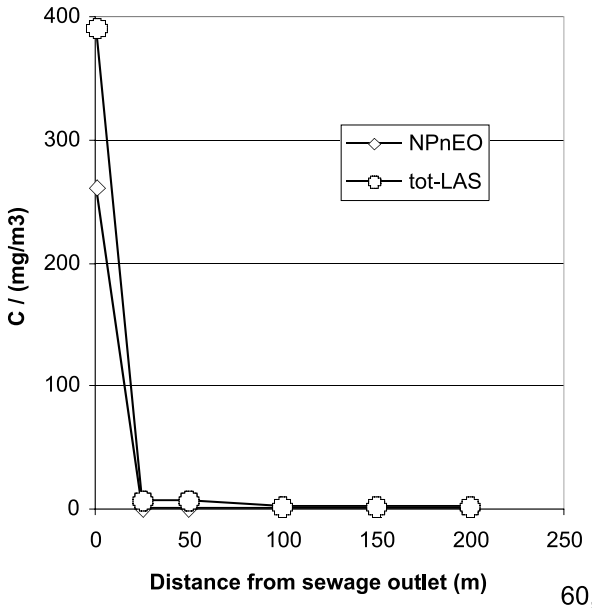

Fig. 4.
Dilution of nonylphenol polyethoxylates (NPnEO) and linear alkylbenzene sulphonates (LAS) in the wastewater plume near the outlet in the Sibenik Harbour.

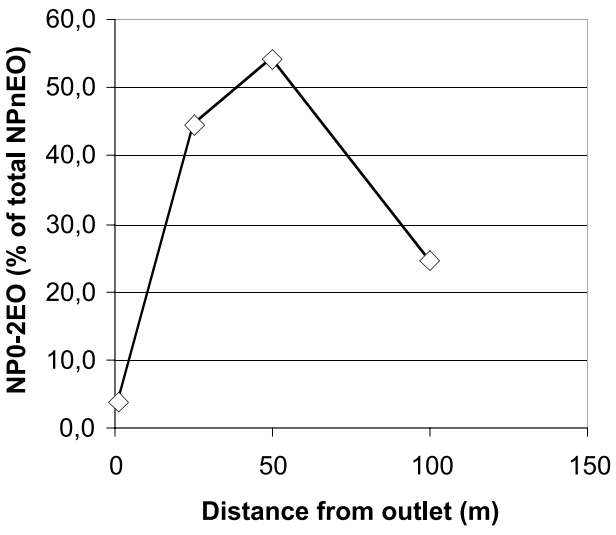

Fig. 5.
Relationship between the parent nonylphenol polyethoxylates (NPnEO) and their lipohylic metabolites (NP0-2) in the wastewater plume in the Sibenik Harbour.

DISTRIBUTION ON THE VERTICAL PROFILE

The most characteristic feature of the surfactant distribution in a microtidal estuary is the distribution pattern on the vertical profile of the water column (Figure 6). The maximal concentrations of both NPnEO and LAS are observed at estuarine phase boundaries, i.e. in the surface microlayer (air-brackish water interface) and at the brackish-seawater interface. Such a tendency of aromatic surfactants to accumulate at the phase boundaries is in agreement with their amphiphylic nature. The enrichment factors in the surface microlayer (sampled with the Garrett net) were in the range from 3.2-19.5, while the apparent enrichment at the brackish water-seawater interface was smaller. However, comparatively lower enrichment factors at the brackish water-seawater interface could be just a consequence of insufficient resolution of the procedure that was used for the sampling of organic films at the brackish water-sea water interface (2 cm), as compared to the well-known Garrett net technique. It should be noted that the concentration of both NPnEO and LAS in underlying seawater layer are very low, indicating that their transport through the brackish water-saline water interface is greatly reduced. The interface prevents both the mixing of the two layers as well as a vertical flux of suspended particles (Zutic and Legovic, 1987). It is interesting to note that a similar vertical concentration profile of NPnEO-derived compounds, characterised by a pronounced maximum at the boundary of two physically different water masses, was observed in Lake Geneva, with the maximum situated at the thermocline (Ahel, 1987).

The homologue and/or oligomer compositions of nonylphenolic compounds vary along the vertical profile, suggesting that significant changes occur during the passage through the interface. As indicated in Figure 6, the concentration of lipophylic metabolites of NPnEO shows a very sharp maximum at the halocline, reaching a high share of over 60 % of the total nonylphenolic compounds. These results indicate that the marine interfaces can be regarded as the critical sites of enhanced exposure to the lipophylic metabolites, which represent the most toxic and endocrine-disrupting species derived from NPnEO surfactants.

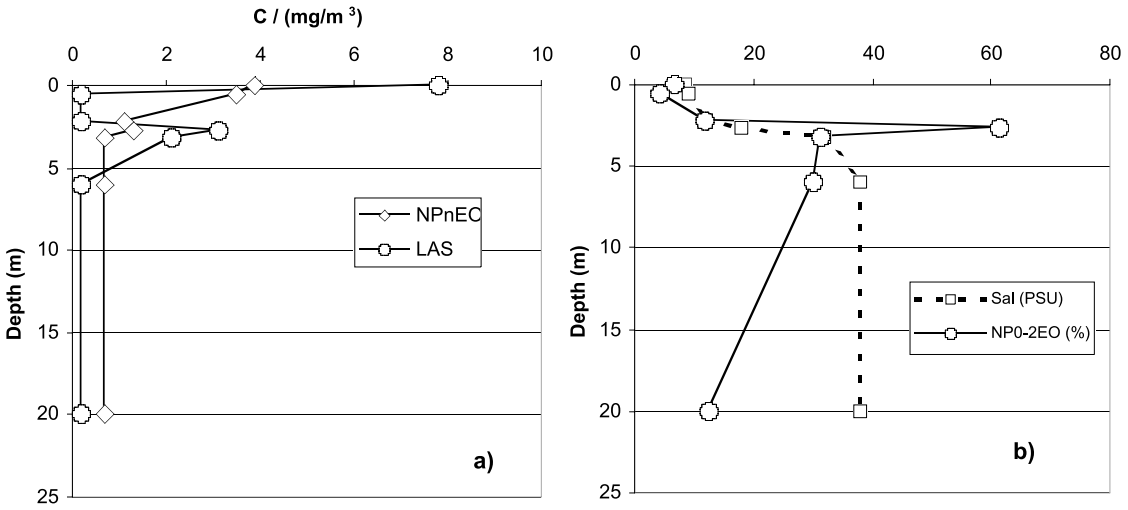

Fig. 6. Vertical profiles of surfactant residues in the water column of a stratified estuary: (a) distribution of nonylphenol polyethoxylates (NPnEO) and linear alkylbenzene sulphonates (LAS) and (b) percentage of lipophylic metabolites in the total NPnEO.

BIOTRANSFORMATION OF AROMATIC SURFACTANTS

The biotransformation processes were shown to be seasonally dependent and the seasonal differences are more clearly reflected in the oligomer composition than in the homologue patterns. For NPnEO oligomers, one could clearly separate the effects of biotransformation and adsorption, since biotransformation is more effective for the higher oligomers, while the lower oligomers exhibit a stronger affinity for adsorption. The corresponding average number of EO

units in summer and winter are typically found in the ranges of 7 to 9 and 9 to 11, respectively, which indicates a more efficient degradation during summer. Field observations on seasonally dependent transformation were confirmed by controlled laboratory experiments, which were conducted using natural microbial populations, originating from both brackish and saline estuarine compartments. The results presented in Table 1 show the estimated biotransformation rates of NPnEO in comparison to those for LAS under different temperature conditions. Estimated overall biotransformation rate constants for NPnEO (0.01 to 0.28 $days^{-1}$) are generally in a similar range like those for LAS; however, a more detailed analysis shows that NPnEO are more resistant to biodegradation by microbial populations from the saline layer and/or under lower temperatures. In addition, the model experiments have showed that biotransformation of NPnEO results in the formation of persistent metabolites, including NP1EO and NP2EO, as well as corresponding carboxylic acids (Kvestak and Ahel, 1995). Since both surfactant classes are biotransformed several times faster by estuarine populations from the brackish layer than those from the underlying saline layer, it was suggested that the present surfactant-degrading bacteria were predominately of wastewater origin (Terzić *et al*., 1992).

The effect of temperature on the biotransformation rate is rather pronounced. At the temperatures representative of summer (23 °C), the transformation rate of LAS is approximately 2 times faster than under winter temperature (14 °C) conditions. This effect is even more pronounced for NPnEO, mainly because of its very slow biotransformation under winter temperature conditions. The biotransformation is particularly inhibited at low temperatures in the saline layer, resulting in very long half-lives in that layer.

Table 1. Overall biotransformation rate constants for nonylphenol polyethoxylates (NPnEO) and linear alkylbenzene sulphonates (LAS) in brackish and saline compartments of the Sibenik Harbour.

Estuarine compartment	Temperature (°C)	NPnEO	LAS
		Rate constant (day^{-1})	Rate constant (day^{-1})
Brackish	13-14	0.02-0.03	0.161
	22.5-23	0.17-0.28	0.247
Saline	13-14	0.01-0.02	0.020
	22.5-23	0.02-0.05	0.156

ASSESSMENT OF ELIMINATION MECHANISMS AND RESIDENCE TIMES IN THE ESTUARY AND POSSIBLE IMPACT ON COASTAL WATERS

To assess the elimination and residence time of NPnEO surfactants, a simple calculation has been performed assuming that biodegradation and transport to the bottom sediment are the main elimination mechanisms. Taking into account a rather low concentration of aromatic surfactants in the surface sediments (<5 mg/kg), the transfer of surfactants to estuarine sediments was calculated to be less than 1 % of the total amount that enters the Sibenik Harbour. Therefore, this mechanism is not expected to be dominant for the removal of aromatic surfactants from the water column. The reason is, most probably, the hydrographic structure of the stratified estuary, which prevents an efficient transport of sediments to the bottom. Consequently, biotransformation should be considered the most important removal mechanism. The efficiency of elimination of aromatic surfactants by biotransformation was estimated using the one-box steady-state estuarine model proposed by Morris (1990). The original expression was modified to suit an irreversible first-order reaction as follows:

$$\text{Biodegradation (\%)} = kT / (1+kT),$$

where k and T represent the biotransformation rate constants and hydrodynamic residence time (flushing time), respectively. The average residence time of water in the brackish layer of the Sibenik Harbour was estimated at 1.2 days, while the residence time of the saline layer is much longer and varies from 6.2 days in winter to 18 days in summer (Legovic, 1991). Taking into account the overall biotransformation rates given in Table 1, it was estimated that the expected removal rate of NPnEO in the brackish layer ranges between 10 % in winter and 25 % in summer. In the saline layer, the elimination of NPnEO in winter is rather restricted (6%), but reaches 47 %

in summer. Most probable explanation for the incomplete elimination of aromatic surfactants in the brackish layer is a very short flushing time of that estuarine compartment, which does not provide enough time for the action of active bacterial populations. On the other hand, biotransformation kinetics of surfactants in the saline water layer is very slow, particularly in winter.

Based on this estimate, it is apparent that most of the surfactant residues entering the estuary are discharged into the coastal waters, though in a very dilute form. Results of a very recent screening of alkylphenolic compounds in Croatian coastal waters are shown in Table 2. As can be seen, the concentrations were found in the ng/L range and even the total concentrations never exceeded 1 μg/L. The most abundant individual class are alkylphenoxy carboxylic acids (NP1EC and NP2EC).

Table 2. Determination of endocrine-disrupting alkylphenolic compounds in Croatian coastal waters (October 2003; concentrations in ng/L).

Location	NP1EO	NP2EO	OP	NP	BPA	OP1EC	NP1EC	NP2EC
Fazana	<30	15	<10	20	10	<10	20	50
Pula	<30	56	<10	50	10	10	280	400
Rijeka	<30	67	<10	20	10	<10	120	230
Bakar	<30	<10	<10	<10	10	10	70	40
Srima	<30	14	<10	20	<10	<10	80	180
Vranjic	<30	<10	<10	<10	<10	<10	50	50
Stobrec	<30	15	<10	30	10	<10	50	40
Gruz	<30	<10	<10	<10	<10	<10	10	<10

CONCLUSIONS

Endocrine-disrupting alkylphenolic compounds are ubiquitous contaminants in municipal wastewaters; consequently, they can be found in all estuarine and coastal waters. At present, it seems that their concentrations in the water column of Croatian coastal waters are rather low and should not pose a direct threat to the fish populations. However, special attention should be paid to estuarine boundaries, where the concentration of the most toxic metabolites can be an order of magnitude higher than that in the bulk of the water column. Since estuarine interfaces represent sites of intensive biological activity, enhanced exposure concentrations in these compartments can be of major environmental concern.

Study of processes governing the fate of organic pollutants in the Eastern Mediterranean

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INTRODUCTION

The present work concerns a comparative study of various atmospheric processes governing the fate of polycyclic aromatic hydrocarbons (Paths), polychlorinated biphenyls (PCBs) and linear alkyl benzene sulphonates (LAS) in Eastern Mediterranean which is a typical subtropical region.

Studies of atmospheric and transport processes of PAHs, PCBs and LAS in the Eastern Mediterranean marine environment, as well as intensive seasonal samplings, were performed from 1999-2002. Air and atmospheric deposition samples were collected at Finokalia, a marine background sampling station (35º 20' N, 25º 40' E, 130 m above the sea level), and the city of Heraklion at the north coast of the Island of Crete, as well as various sites along the Aegean Sea. Sediment trap samples were collected at a site in Eastern Mediterranean west of Crete. Thus, the role of atmospheric deposition as a pathway of pollutants to the deep Mediterranean environment was assessed based on measurements of PAHs and PCBs in the sediment traps deployed in the Eastern Mediterranean, and via comparison between the import (atmospheric deposition of pollutants) and export fluxes (fluxes measured using sediment traps).

The role of water-soluble organic compounds (WSOC) on cloud microphysics has been reported very recently. In particular, WSOC have been characterized as surfactants with a crucial influence on cloud condensation nuclei (CCN) formation. Linear alkylbenzene sulphonates (LAS) are widely used as surfactants in many industrial and household applications, but they have been reported only in aquatic environments. In this study, the occurrence of LAS in background and urban coastal atmosphere is reported for the first time.

Although the above mentioned compound classes can be considered as "old" contaminants, we demonstrate that the biogeochemical processes concerning these substances in the marine environment remain a field of research to explore. In addition, the results of our study, particularly on LAS, might be applicable in designing any future study on the fate of novel contaminants such as pharmaceuticals in the coastal Mediterranean environment.

METHODS

Sampling procedures for PAHs and PCBs are described in Manadalakis and Stephanou (2002), Tsapakis and Stephanou (2003) and Tsapakis *et al*. (2003).

The analysis of samples for the determination of PAHs was preformed according to Gogou *et al*. (1998). For the determination of PCBs, samples were treated and analyzed according to Mandalakis *et al*. (2001).

The analysis for the determination of LAS in coastal waters and atmospheric samples was conducted according to Markoulakis (2004).

RESULTS AND DISCUSSION

Table 1 recapitulates the range and mean concentration of PAHs and PCBs measured in the aerosol, gaseous and deposition (wet and dry) samples collected at Finokalia.

Occurrence of PAHs and PCBs air and atmospheric deposition samples in Eastern Mediterranean Sea.

Total atmospheric concentration of PAHs in Finokalia, varied form 4.14 to 57.16 ng m^{-3}, and gas phase PAHs contribute 90% or more of total atmospheric levels. Long-range transport was determined to be the major source of PAHs in the eastern Mediterranean atmosphere. Atmospheric samples with the highest concentrations of PAHs originated in eastern and central Europe. Concentration of gas phase PAHs was equally distributed over the eastern Mediterranean Sea while particulate phase was significantly higher close to urban areas.

Table 1. Range and mean concentration of PAHs and PCBs measured in gaseous, particulate and deposition samples.

Compound Class	Concentration Gas (ng m^{-3})	Concentration Particles (ng m^{-3})	Wet Deposition (μg m^{-2} d^{-1})	Dry Deposition (μg m^{-2} d^{-1})
∑PAHs	3.6-52.6	0.4-1.7	0.13-0.90	0.03-0.26
	(pg m^{-3})	(pg m^{-3})	(ng m^{-2} d^{-1})	(ng m^{-2} d^{-1})
∑PCBs	41-397	0.7-9.4	0.1-5.6	0.1-1.0

PAHs flux due to air/ sea exchange was estimated to be 693 μg m^{-2} y^{-1} in the whole eastern Mediterranean basin. Total concentration of PAHs in wet deposition samples was 343 ng l^{-1}. Dry deposition flux for PAHs was 0.18 μg m^{-2} y^{-1}.

The average concentrations of total PCB congeners (∑PCBs) in the gas and particulate phase of the atmosphere were 68.1±28.8 pg/m^3 and 2.3±1.8 pg/m^3, respectively. The lack of seasonal variation for the atmospheric concentration of individual congeners and ∑PCBs and the shallow slopes obtained from the Clausius-Clapeyron plots for several PCB congeners indicated that long-range transport is the main factor controlling the atmospheric levels of PCBs in this area. Most of the episodes with elevated concentrations of ∑PCBs concurred with air transport from Western and Central Europe.

The average concentration of ∑PCB (sum of 54 PCB congeners) in precipitation samples collected from Finokalia station was 1.9±0.9 ng l^{-1}. The percentage of particle-bound PCBs ranged between 11 and 53%, providing an average value of only 30±16%. Based on these data, it was deduced that the annual wet deposition flux of PCBs should approach 832 ng m^{-2} $year^{-1}$. The dry deposition flux of ∑PCB ranged between 39 and 394 ng m^{-2} $year^{-1}$, with an average value of 179±125 ng m^{-2} $year^{-1}$. Our results suggested that the annual dry deposition flux of particulate PCBs in eastern Mediterranean should be about 4.5 times lower than the wet deposition flux of these chemicals.

Reactions of PAHs and PCBs with atmospheric oxidants

Gas phase reaction of PAHs with OH radicals is an important production process of the highly mutagenic and carcinogenic nitro-PAHs. During a three-day intensive measurement campaign in Finokalia (summer 2001), the concentration of 2-NF and 2-NP varied from 3.35 to 78.88 pg m^{-3} and from 2.22 to 21.98 pg m^{-3}, respectively. The highest concentrations were determined during noon hours, characteristic of reactions between gas PAHs with OH radicals

The variation of PCB total concentration, during the above-mentioned intensive measurement campaign in Finokalia, showed a diurnal pattern inversely related to that of OH (Figure 1). The

diurnal variation of PCBs observed in our study suggested that the daytime depletion observed for the PCBs should be attributed to their reaction with OH radicals. Figures 1a, b and c present the diurnal variation of the total PCB concentration (∑PCBs, sum of 27 congeners). The simultaneous variation of the OH radical concentration for each of the three intensive sampling periods is also shown in the same figures. In all cases, the variation of ∑PCBs concentration showed a diurnal pattern inversely related to that of OH. In particular, the highest OH radical concentrations were observed approximately between 10:00 and 15:00 EEST (Eastern European Summer Time), while the lowest ∑PCBs concentrations were measured during the same daytime period (Figures 1a,b,c). Figures 1d, e and f show the corresponding diurnal variation of the ambient temperature. As for OH radical concentration, an opposite trend between ambient temperature and ∑PCBs concentration was observed. The diurnal variation of PCBs observed in our study suggests that the volatilization/exchange of PCBs from contaminated surfaces was of minor importance.

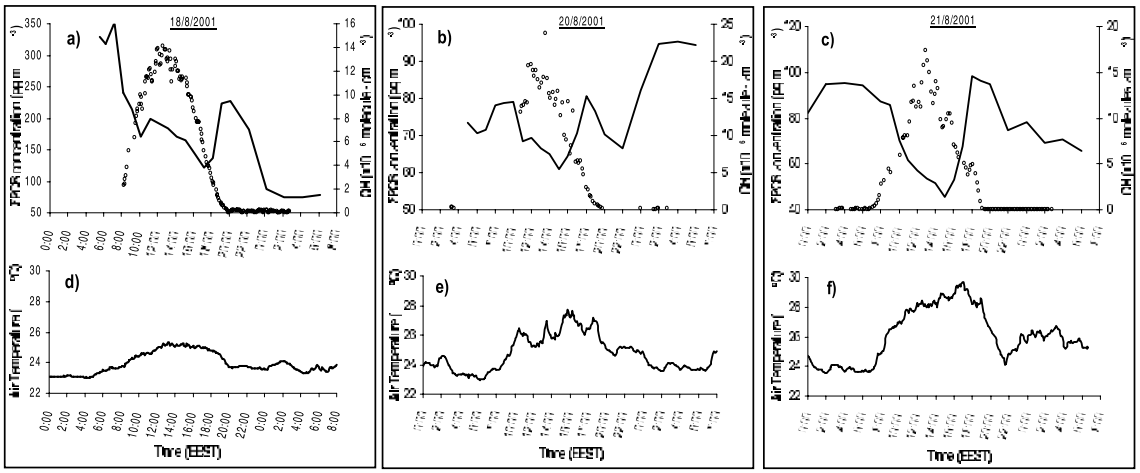

Fig. 1. Diurnal variation of OH radicals (dotted line) and PCBs (continuous line) in the Eastern Mediterranean atmosphere.

Budget of PAHs and PCBs in Eastern Mediterranean Sea

One-box budget can be constructed for PAHs. Based on field data, we have estimated that 235 tonnes y^{-1} and 75 tonnes y^{-1} are eliminated from the atmosphere through wet and dry deposition, respectively. Air-water exchange flux (F_{A-W}) estimation has indicated air transport as a significant source of PAHs to pristine marine sediments of Eastern Mediterranean (1644 tonnes y^{-1}; Figure 2). Calculations on the basis of the above data have shown that ca. 1954 tonnes y^{-1} of PAHs should be transported to the area from N, NW, central and NE Europe.

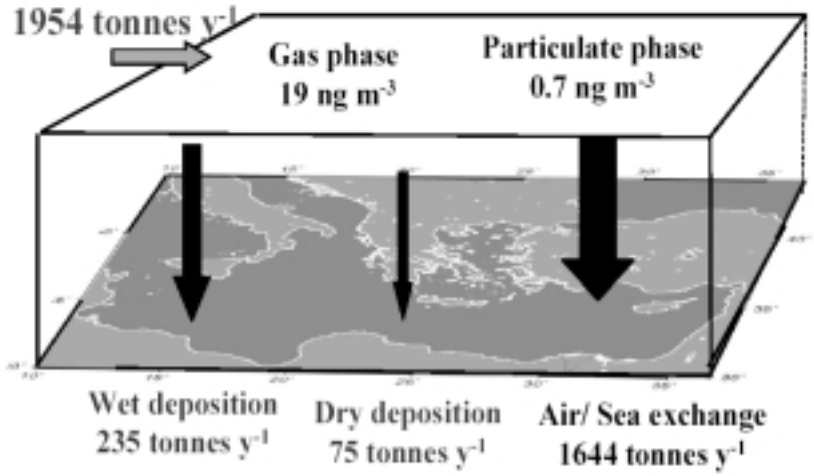

Fig. 2. One-Box model of PAHs in the Eastern Mediterranean.

For PCBs calculations similar to those for PAHs, field data have shown that 1,120 kg y^{-1} and 220 kg y^{-1} are eliminated from the atmosphere through wet and dry deposition respectively. Air-sea exchange was responsible for the elimination of 705 kg y^{-1}, while PCB losses via hydroxyl radicals in the atmosphere may be responsible for the elimination of 4,000 kg y^{-1} (Figure 3). Thus, 6080 kg y-1 might be introduced to the area from N, NW, central and NE Europe.

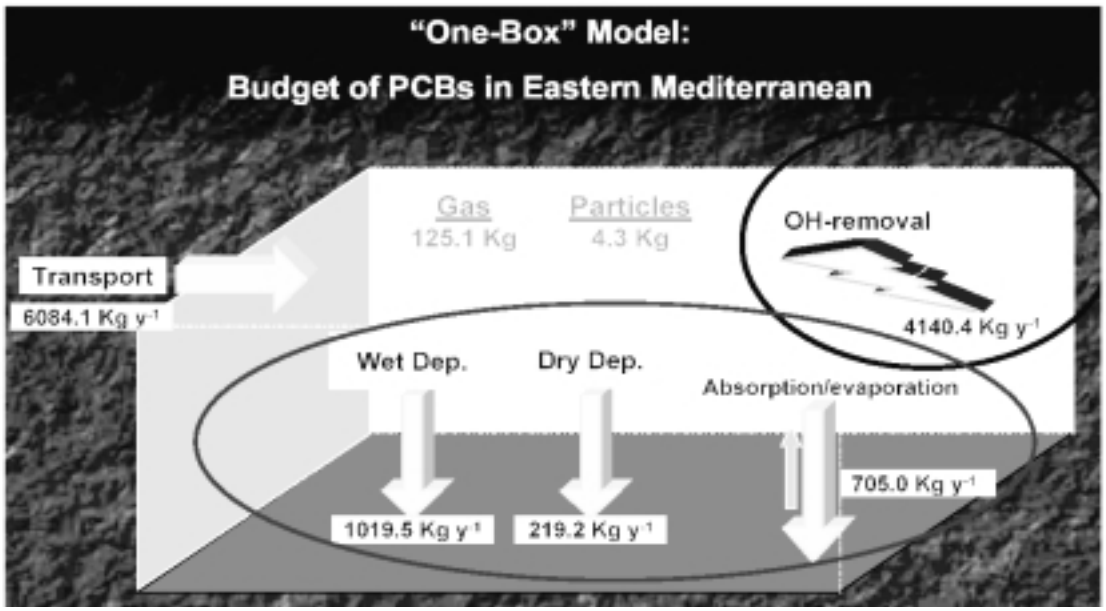

Fig. 3. One-Box model of PCBs in the Eastern Mediterranean.

PAH and PCB fluxes determined in the sediment traps

Sediment traps deployed in the Eastern Mediterranean have shown fluxes for total PAHs and PCBs at depths (250-2820 m) ranging from 10-8 μg m^{-2} y^{-1} and 210 - 140 ng m^{-2} y^{-1}, respectively (Figure 4).

Comparison of these values to those reported on the atmospheric deposition pathway (for PAHs 959 μg m^{-2} y^{-1} and for PCBs 1590 ng m^{-2} y^{-1}; Figure 4) clearly indicates that the majority of

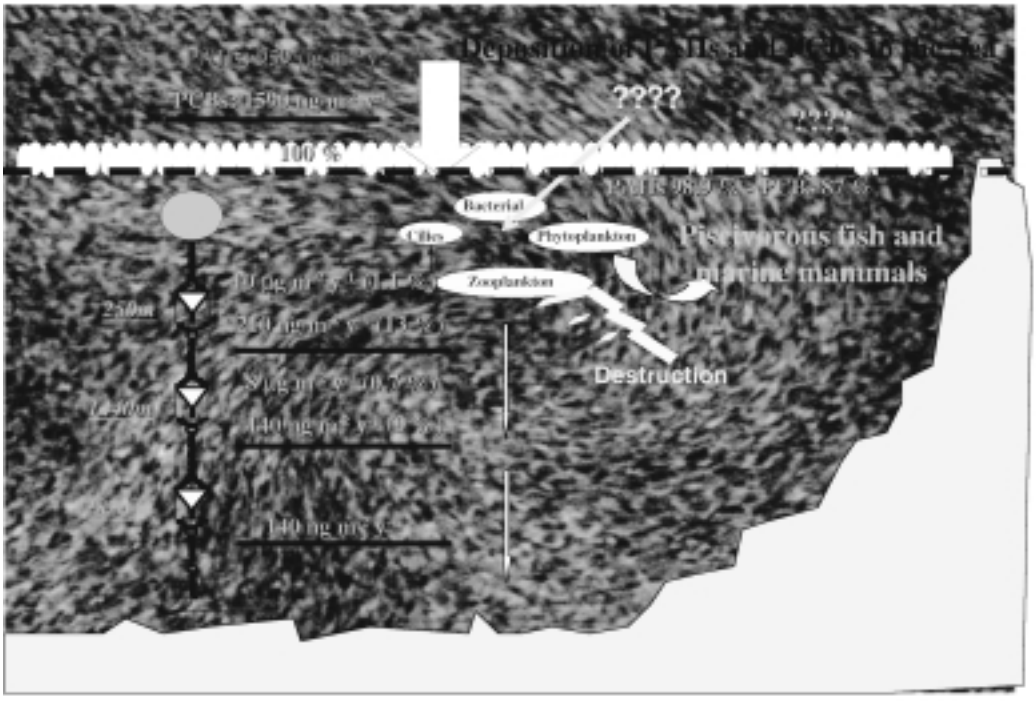

Fig. 4. Sediment fluxes of PAHs and PCBs in the Eastern Mediterranean water column.

PAHs (98.9%) and PCBs (87%) is remaining in the euphotic zone, where it could be either destroyed or assimilated by piscivorous fishes and marine mammals.

Presence of LAS in the coastal atmosphere

Water-to-air volatilization of LAS from coastal sea waters and aeration tanks of wastewater treatment plants (WWTP) are sources of LAS to the coastal atmosphere. Furthermore, the high concentrations found in the coastal atmosphere (8-200 ng m^{-3} in urban areas and 2-4 ng m^{-3} in background areas) suggest that anthropogenic surfactants might be an important component of atmospheric organic carbon. HPLC and HPLC/MS analyses have shown that atmospheric LAS exhibit a pattern similar to that determined in coastal sea water. In addition, their atmospheric concentrations increase with increasing wind speed. Cascade impactor air sampling has shown that LAS are mostly associated with aerosol particles having diameters < 1mm, suggesting long atmospheric residence time. In bulk deposition samples, the concentration of LAS varied from 2 to 6 μg L^{-1}.

Prediction of the environmental load of pharmaceuticals contaminating the marine environment. A case for the Adriatic Sea.

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ABSTRACT

The recent scientific literature is rich in studies of pharmaceuticals in effluents of sewage treatment plants, and surface and ground water in Europe and North America, but information on the marine environment is still scanty. Attempts at risk assessment in fresh water are being made, but exposure data for the marine environment are lacking. This paper provides an estimate of the types and amounts of pharmaceuticals entering the Adriatic Sea from the River Po, using data from a recent study of environmental concentrations of pharmaceuticals in this river. After prioritisation according to theoretical environmental loads, the pharmaceuticals were measured in water samples collected near the mouth of the River Po in the Adriatic Sea. Concentrations were multiplied by the annual flow rate of the river, providing a gross estimate of the loads of the pharmaceuticals flowing annually into the sea. This preliminary prioritisation of pharmaceuticals of concern for the coastal environment of the Adriatic Sea identified a short list of putative molecules, such as atenolol, erythromycin, furosemide, and ranitidine, and highlighted the need for further research on another group of pharmaceuticals, including lincomycin, clarithromycin, hydrochlorothiazide and salbutamol.

INTRODUCTION

Until recently, information on medicinal substances released into the environment was scant but there are now numerous studies measuring pharmaceuticals in effluents of sewage treatment plants (STPs) and surface and ground water in Europe and North America (Heberer *et al*., 1998; Metcalfe *et al*., 2003). Several thousand tons of medicinally active substances are used every year in the world for humans and animals, and excreted as parent compounds or active metabolites, escaping degradation in STPs, and entering the fresh water environment. Knowledge on this topic is rapidly growing and information on the types and concentrations of the pharmaceuticals contaminating rivers and ground water serves to assess risks in fresh water (Stuer-Lauridsen *et al*., 2000; Daughton and Ternes, 1999).

While this information is sufficient for initial risk assessment, our knowledge for the marine environment is still scanty, and only few studies have measured concentrations of a limited number of medicinal substances in the North Sea (Buser *et al*., 1998; Weigel *et al*., 2002).

Environmental concentrations of the pharmaceuticals are generally in the μg/L range in STP effluents and ng/L in rivers and ground water. Therefore because of dilution or degradation, the levels of the pharmaceuticals in the sea might be very low. However, pharmaceuticals are designed to stimulate a response in humans and animals at low doses, with very specific targets, and we have little information on their effects on non-target organisms. The possibility of effects on marine living organisms cannot therefore be dismissed, even at low concentrations. Thus, implications for the marine environment need to be assessed.

The first step is to obtain exposure figures. Here we report the findings of an exercise to predict the types and amounts of pharmaceuticals entering the Adriatic Sea from the River Po, the major Italian river with a drainage basin of about 18 million inhabitants, in the most industrialised area of Italy. This exercise used a dataset from our recent work of prioritisation, calculation of the predicted concentrations, and measurement of the environmental concentrations of pharmaceuticals in the River Po (Calamari *et al*., 2003).

MATERIALS AND METHODS

The River Po

The Po is the main Italian river, flowing from the Alps to the Adriatic Sea, with an average and a maximum flow rate at the mouth of 1500 and 10,000 m^3/s, respectively. It collects wastewater from a catchment area of about 70,000 km^2, with about 18 million people, in the most densely inhabited and industrialized areas of Italy. The River Po drains sewage from about half the animal settlements in Italy. In previous studies we analysed water and sediments from seven sampling stations along the river, located after the inlets of the main affluent, and downstream of the major towns. In the present study we used the dataset obtained in Pieve Saliceto to predict loads of the pharmaceuticals flowing into the Adriatic Sea. Pieve Saliceto was the terminal sampling site (number 7, 460 km from the source, flow rate 1085 m^3/s, 12.3 million inhabitants in the drainage basin), selected to measure the load from the whole drainage basin. Distances from the source and number of inhabitants were from I.R.S.A (I.R.S.A., 1977). The annual flow rates were provided by the "Ufficio Mareografico ed Idrografico del Po". Other data are from "Idrografia e Idrologia del Po" (Cati, 1981).

Prioritisation of pharmaceuticals

To monitor pharmaceuticals in the environment we chose an approach based on preliminary screening of the molecules most likely to cause environmental problems. The prioritisation was based on the theoretical environmental loads, which in turn were calculated by multiplying sales figures by the rate of metabolism in man. We used official Ministry of Health prescription data to estimate sales. By correcting for the percentage of excretion as parent compound after administration in man, annual sales figures were converted into theoretical environmental loads. Only the drugs with the highest theoretical loads were considered for analysis in the environment. A group of widely used veterinary drugs was also considered, to provide a figure for contamination by veterinary pharmaceuticals.

Measurement of pharmaceuticals in water

The pharmaceuticals were measured in water samples collected from the River Po at the Pieve Saliceto sampling site, *i.e*. near the mouth of the river in the Adriatic Sea. The pharmaceuticals belong to various therapeutic categories and have a wide range of chemical structures and properties. A method for their analysis must be sensitive, versatile and specific enough to measure the molecules in the ng/L range, must include all the candidate pharmaceuticals, and provide reliable data for each single molecule. For these reasons we chose solid-phase extraction (SPE) and HPLC-MS-MS analysis.

After preliminary investigations, pharmaceuticals were divided in three groups, according to their physical-chemical properties, and three different SPE methods were selected for their extraction: an OASIS MCX cartridge (60 mg, Waters Corp, Milford, MA, USA), a LichrolutEN cartridge (200 mg, Merck, Darmstadt, Germany), and a Bakerbond C_{18} cartridge (500 mg, Baker, Phillipsburg NJ, USA). After extraction and purification, samples were analysed with a triple-quadrupole HPLC-MS-MS system consisting of two Perkin-Elmer Series 200 pumps, a Perkin-

Elmer Series 200 auto-sampler and an API 3000 mass spectrometer (Applied Biosystem-Sciex, Toronto, Canada). The mass spectrometer was equipped with a standard Turbo Ion Spray ionisation source. HPLC separation was done with a LUNA C8 column 50 mm × 2 mm i.d., 3 μm particle size (Phenomenex, Torrance, CA, USA). The analysis was done in positive and negative ionisation mode, depending on the substance to be analysed. Details of the analytical method are reported elsewhere (Castiglioni *et al*., 2004).

Load of pharmaceuticals flowing into the Adriatic Sea

The concentrations of the pharmaceuticals measured in the water sample from the River Po collected in Pieve Saliceto, *i.e*. the terminal sampling site near the mouth of the river in the Adriatic Sea, were multiplied by the annual flow rate of the river to obtain a gross estimate of the loads of pharmaceuticals flowing annually from the River Po into the Adriatic Sea.

RESULTS AND DISCUSSION

To obtain a gross estimate of the load of some priority pharmaceuticals entering the Adriatic Sea from the River Po, we multiplied concentrations in the river water at its mouth by the annual flow rate at the sampling site. Results of this extrapolation, shown in Table 1, provide an estimate of the order of magnitude of the contamination. Obviously, we also need to consider dilution and degradation rates in order to attain a figure of the concentrations in coastal water. The degradation rate of the pharmaceuticals in marine water is largely unknown, and can only be extrapolated from degradation data in fresh water or in laboratory conditions, sometimes available from the literature (Castiglioni *et al*., 2004). Data are summarised in Table 2, which show that some pharmaceuticals have short half-lives in the environment and are rapidly degraded (amoxycillin, ceftriaxone, ibuprofen, bezafibrate, omeprazole), while others are reported to be stable in fresh water for longer periods (atenolol, ciprofloxacin, enalapril, erythromycin, furosemide, ranitidine). No data were available for other potentially relevant molecules, such as lincomycin, clarithromycin, hydrochlorothiazide, salbutamol (Concannon *et al*., 1986; Teraoka *et al*., 1993).

Table 1. Pharmaceuticals measured in the River Po (sampling site Pieve Saliceto, River flow rate 1085 m³/s, concentrations in ng/L, [Calamari *et al*., 2003]) and predicted loads of the pharmaceuticals flowing annually from the River Po into the Adriatic Sea (kg/year).

Pharmaceuticals	*Concentration in River Po (ng/L)*	*Annual load in the Adriatic Sea (kg/year)*
amoxycillin	nd	-
atenolol	17.23	589.3
bezafibrate	2.30	78.7
ceftriaxone	nd	-
ciprofloxacin	nd	-
clarithromycin	1.67	57.1
cyclophosphamide	nd	-
diazepam	0.83	28.4
enalapril	0.05	1.71
erythromycin	2.75	94.0
furosemide	3.48	119.0
ibuprofen	7.23	247.3
hydrochlorothiazide	9.73	332.8
omeprazole	nd	-
ranitidine	1.60	54.7
spiramycin	nd	-
lincomycin	139.40	4767.5
oleandomycin	0.08	2.8
oxytetracycline	0.19	6.5
salbutamol	1.56	53.4
tilmicosin	nd	-
tylosin	0.30	10.3

If this is confirmed in marine water, one might predict an accumulation in coastal water of the medicinal substances with long half-lives and high concentrations in river water (atenolol, erythromycin, furosemide, ranitidine). Unfortunately, we have no data to predict the environmental behaviour of several of the pharmaceuticals detected in the river, and cannot therefore estimate their potential for accumulation. This is true in particular for lincomycin; because of its high load, probably due to its extensive use in animals (Calamari *et al*., 2003), might be of some concern for the coastal environment. Absence of data for environmental behavior prediction also exists for clarithromycin, hydrochlorothiazide, and salbutamol.

In conclusion this exercise was done for a preliminary prioritisation of the pharmaceuticals of concern for the coastal marine environment of the Italian side of the Adriatic Sea. This identified a short list of molecules, such as atenolol, erythromycin, furosemide and ranitidine and suggested the need for further research on molecules such as lincomycin, clarithromycin, hydrochlorothiazide and salbutamol.

Table 2. Stability of the pharmaceuticals in water (Castiglioni *et al.,* 2004).

Pharmaceuticals	***Stability in water***	***References***	***Comments***
Amoxycillin	t_{90} < 2 d	15	Unstable in water
Atenolol	stable for 40 d (5-25°C)	16	
	t_{50} 45.2 h pH 7.4 (UV ray)	17	
Bezafibrate	83% degraded in 6 d in STP	18	
Ceftriaxone	t_{90} 250 h (pH 6, 20 °C)	19	
Ciprofloxacin	stability > 40 d in closed bottle test		
	t_{50} 90.2 min (xenon lamp 200W/m^2)	20	
	t_{50} 1.6-2.5 d in STP	21	
Clarithromycin	t_{50} 1.3 h pH 2, 37°C	22	Excessively acidic conditions
	t_{50} 17 min pH 1.39	23	Excessively acidic conditions
Enalapril	Stable 56 d (25°C)	24	
	Stable 91 d (4 °C)	24	
Erythromycin	t_{50} ≥ 1 y	25	
	11.5 d (20°C)	26	
	t_{50} 3 s pH 1.39	23	
Furosemide	Stable 90 d pH 5.2	27	
	Stable 96% 240 d pH 5.2	27	
Hydrochlorothiazide	-		No data
Ibuprofen	t_{50} < 1 d	25	
	90% degraded in 6 d STP	18	
Lincomycin	-		No data
Omeprazole	70% 1-2 d pH 5.9- 7.0	28	
	26% 14 d pH 7.8	28	
	94% > 100 d pH 11	28	
	73% 6d pH 7.0	29	
Ranitidine	Stable 160 h pH 6.18, 65°	30	
Salbutamol	-		No data
Spiramycin	-		No data

Occurrence and fate of antibiotics as trace contaminants in wastewaters, sewage sludges and surface waters

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INTRODUCTION

Human-use pharmaceuticals enter sewage effluents via urine and feces and improper disposal. These pharmaceuticals are discharged from private households and hospitals. In many countries, sewage effluents usually reach wastewater treatment plants (WWTPs) (Figure 1). However, direct inputs into natural waters are also possible during rain events, particularly in the case of less industrialized countries. Antibiotics are only partially eliminated in wastewater treatment plants, and residual amounts can reach ambient waters or groundwater. Most pharmaceuticals are found in natural waters at only very low concentrations. Despite this general finding, there arises the question of potential risks posed by traces of pharmaceuticals on aquatic ecosystems. Antibiotics are of particular interest because we do not currently know whether their presence in natural waters contributes to the spread of antibiotic resistance of microorganisms.

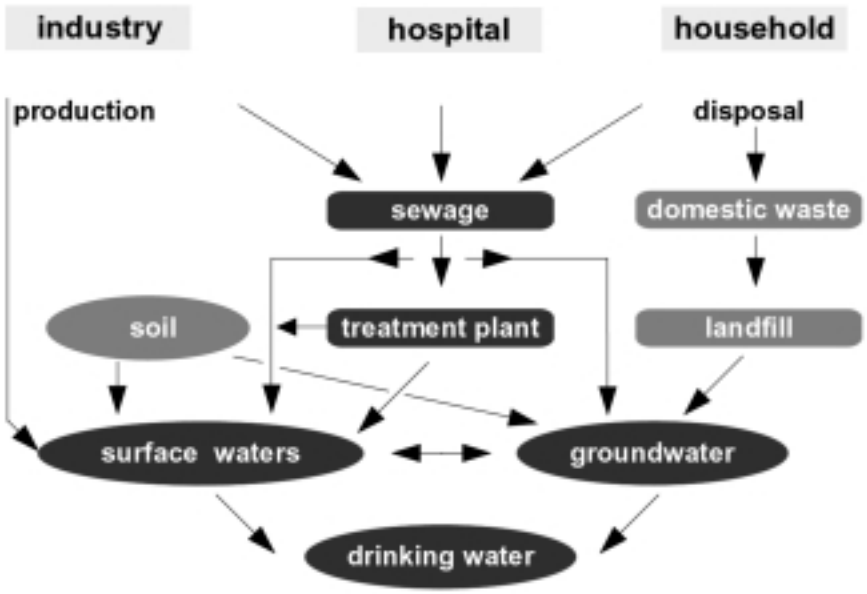

Fig. 1. Exposure routes of human-use antibiotics into wastewaters and the environment.

Hirsch *et al*. (1999) initially investigated the occurrence of several representatives from the main groups of antibiotics in wastewater treatment plant effluents and in river water. They described the analysis of various water samples for 18 antibiotic substances from the antibiotic classes of macrolides, sulfonamides, penicillins and tetracyclines. They observed the frequent occurrence of erythromycin-H_2O, roxithromycin, and sulfamethoxazole with concentrations up to 6 μg/l in WWTP effluents. Neither tetracyclines nor penicillins could be detected at concentration levels above 50 and 20 ng/l, respectively. Penicillins are not very likely to occur in the aquatic environment due to the chemically unstable ß-lactam ring, which is readily susceptible to hydrolytic cleavage. Sacher *et al*. (2001) analyzed 105 groundwater wells in Baden-Wuerttemberg, Germany. Among 60 pharmaceuticals, erythromycin-H_2O and sulfamethoxazole were the only antibiotics out of 8 compounds they detected in at least three groundwater samples. Recently, a study was published (Kolpin *et al*., 2002), which shows the occurrence of 95 organic wastewater contaminants including pharmaceuticals in 139 streams across the USA. Among 31 antibiotics from the groups of tetracyclines, macrolides, sulfonamides, and fluoroquinolones, erythromycin-H_2O and sulfamethoxazole were found in concentrations of up to 1.7 and 1.9 μg/l, respectively.

Here we present an overview report on the current state of our projects encompassing fluoroquinolone and macrolide antibiotics, which are used in human medicine. One preliminary article (Alder *et al*., 2001) and five full reports on our investigations have been published elsewhere (Golet *et al*., 2001, 2002a,b, 2003; McArdell *et al*., 2001). In our early work, we had dealt with human- and veterinary-use antibiotics and demonstrated different input pathways of the two types of antibiotics (Alder *et al*., 2001). Currently, we are focusing on antibiotics that have medicinal applications in hospitals and are also used by individuals.

FLUOROQUINOLONES

An analytical method was developed for the trace enrichment procedure for fluoroquinolones (FQs) from wastewaters and ambient waters. The analytical method based on reverse-phase liquid chromatography was successfully applied to quantify FQs in effluents of urban wastewater treatment (Golet *et al*., 2001). Simultaneous determination was carried out at treatment plants and in ambient waters (Golet *et al*., 2003). Out of the ten investigated compounds, the FQs ciprofloxacin and norfloxacin were found in detectable concentrations. LC/FLD proved to be a compound-specific method that could be utilized to quantitatively analyze wastewater treatment plant effluents. Both ciprofloxacin and norfloxacin are derived from human-use medication, contributing to around 90% of the FQs consumed in Switzerland. Not surprisingly, none of the investigated veterinarian-use FQs were detected in urban wastewater because of the different entry route of veterinary drugs into the environment (i.e., via manure dispersion and animal excretion onto soils). Preceded by an efficient extraction procedure such as accelerated solvent extraction (ASE), the developed method could also serve to determine FQ contents in sewage sludges and in sludge-treated soils (Golet *et al*., 2002b). A 50 mM aqueous phosphoric acid/acetonitrile mixture (1:1) was found to be optimum in combination with an extraction temperature of 100 °C at 100 bar, over a period of 60 and 90 minutes for sewage sludge and sludge-treated soil samples, respectively. A cleanup step using solid-phase extraction substantially improved the selectivity of the method. This method was successfully applied to untreated and anaerobically digested sewage sludges and sludge-treated soils. Ciprofloxacin and norfloxacin were found in sewage sludges from several wastewater treatment plants at concentrations ranging from 1.4 to 2.4 mg/kg of dry matter. Therefore, FQs may reach the terrestrial environment as indicated by the occurrence of FQs in topsoil samples from experimental fields, to which sewage sludge had been applied (Golet *et al*., 2003). Concentrations of FQs were determined in filtered wastewater (raw sewage, primary, secondary, and tertiary effluents) and suspended solids, sewage sludge (raw, excess, and anaerobically digested sludge), and sludge-treated soils. Mass balance results are shown in Figure 2. Wastewater treatment resulted in a reduction of the FQ mass flow of 88–92%, mainly due to sorption on sewage sludge. No significant removal of FQs occurred under methanogenic conditions of the sludge digesters.

Mass flows of FQs were investigated in the aqueous compartments of the Glatt valley watershed, a densely populated region in Switzerland (Golet *et al*., 2003). FQ concentrations and loads were determined in municipal wastewater effluents and in the receiving surface water, the Glatt river.

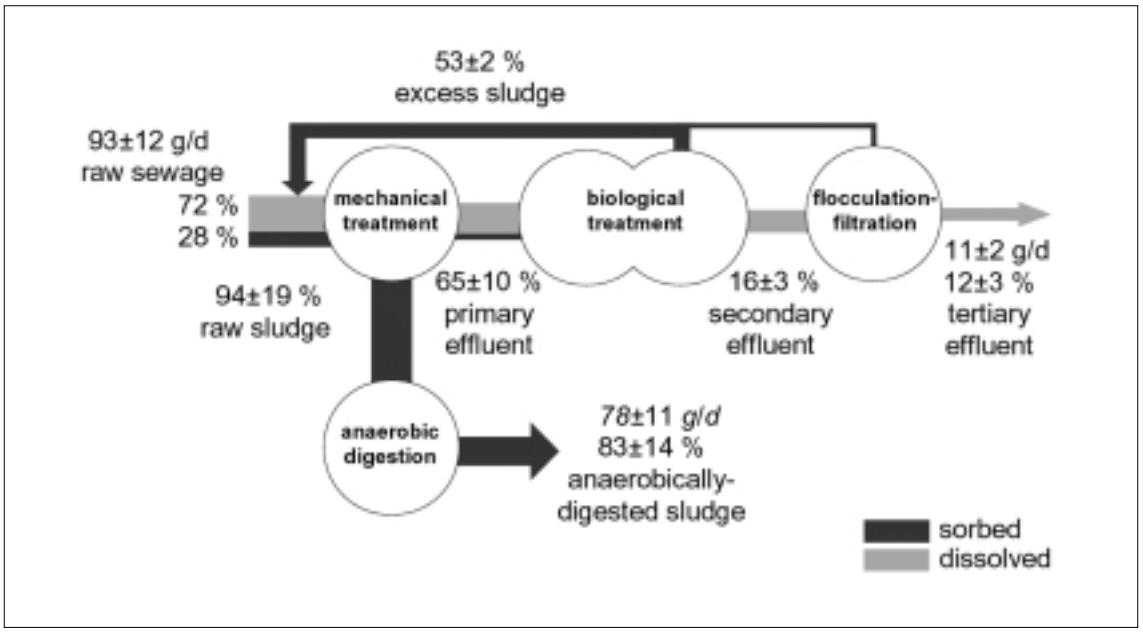

Fig. 2. Absolute daily loads and relative mass transfer of ciprofloxacin through a mechanical-biological wastewater treatment plant (for details see Golet *et al.,* 2003).

Individual concentrations in raw sewage and in final wastewater effluents ranged from 255 to 568 ng/l and from 36 to 106 ng/l, respectively. In the Glatt river, the FQs were present at concentrations below 19 ng/l. The removal of FQs from the water stream during wastewater treatment was between 79 and 87%. During the studied summer period, FQs in the dissolved fraction were significantly reduced downstream in the Glatt river (15–20 h residence time) (66% for ciprofloxacin and 48% for norfloxacin) (Figure 3). Thus, it is demonstrated that an additional decrease in residual levels of FQs in the aquatic environment occurs after wastewater treatment. Figure 4 shows the decrease of the measured concentrations along the exposure route from hospital wastewater to wastewater treatment and finally to river water. The concentrations of ciprofloxacin in grab samples of the hospital outflow were reduced up to two orders of magnitude by dilution before entering the WWTP. The WWTPs proved to be efficient removal barriers for

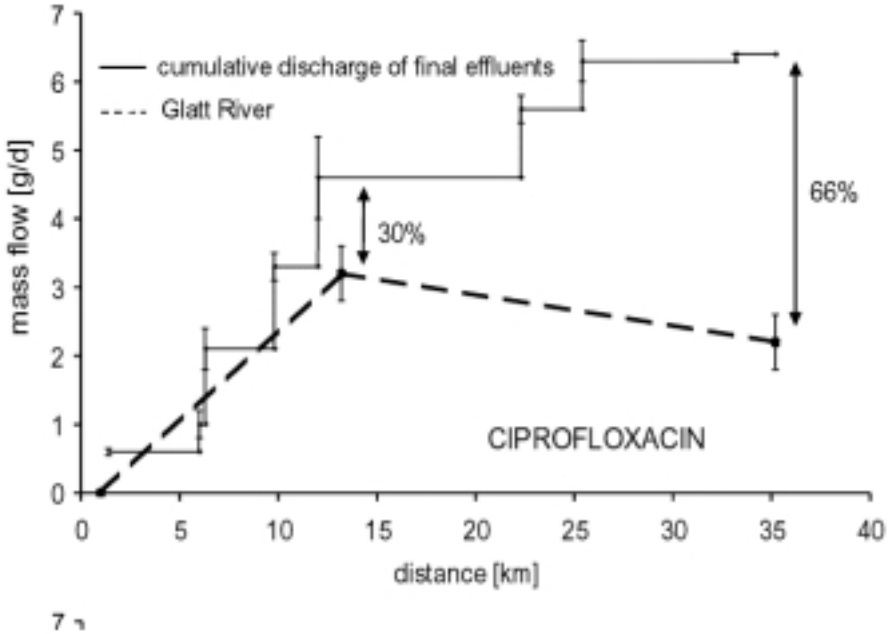

Fig. 3. Longitudinal mass flow profiles of ciprofloxacin in the Glatt river, Switzerland (for details see Golet *et al.,* 2002a).

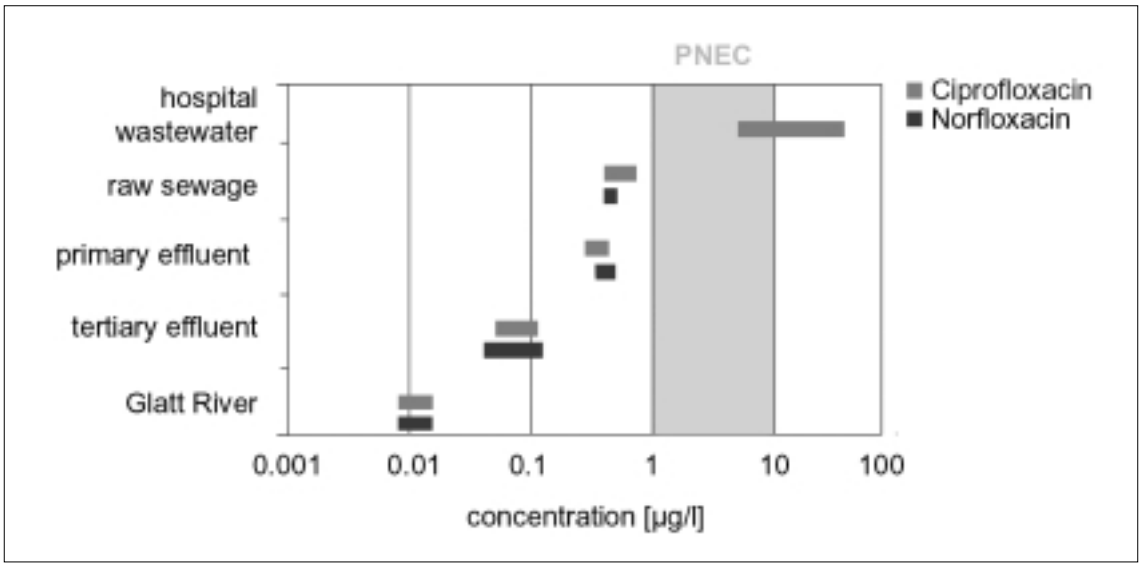

Fig. 4. Concentration ranges and predicted no effect concentrations (PNEC) of ciprofloxacin and norfloxacin in wastewater and in the aquatic environment.

the FQs before entering the Glatt. As mentioned above, the removal of FQs in the WWTPs is mainly due to sorption on sewage sludge.

The exposure data of ciprofloxacin for final effluents and river water were related to acute toxicity for aquatic organisms. Following the recommendations of the European guidelines and draft documents, a predicted no effect concentration (PNEC) of 3 μg/l of the algae *Selenastrum capricornutum* in surface waters was calculated using EC50 (growth inhibition). A PNEC of 8 μg/l was obtained, using EC50 (growth inhibition) data, for a relevant bacterial population of *Pseudomonas putida* in WWTP. These values are comparable to the lowest found minimum inhibition concentration (MICs) for ciprofloxacin (MIC $\geq$ 10 μg/l) without applying further safety factors or with a MIC of 1 μg/l when applying a safety factor of 10, which should explain intraspecies variability. As shown in Figure 4, only the concentrations in hospital wastewaters exceed the calculated PNEC range (risk quotient MEC/PNEC >1).

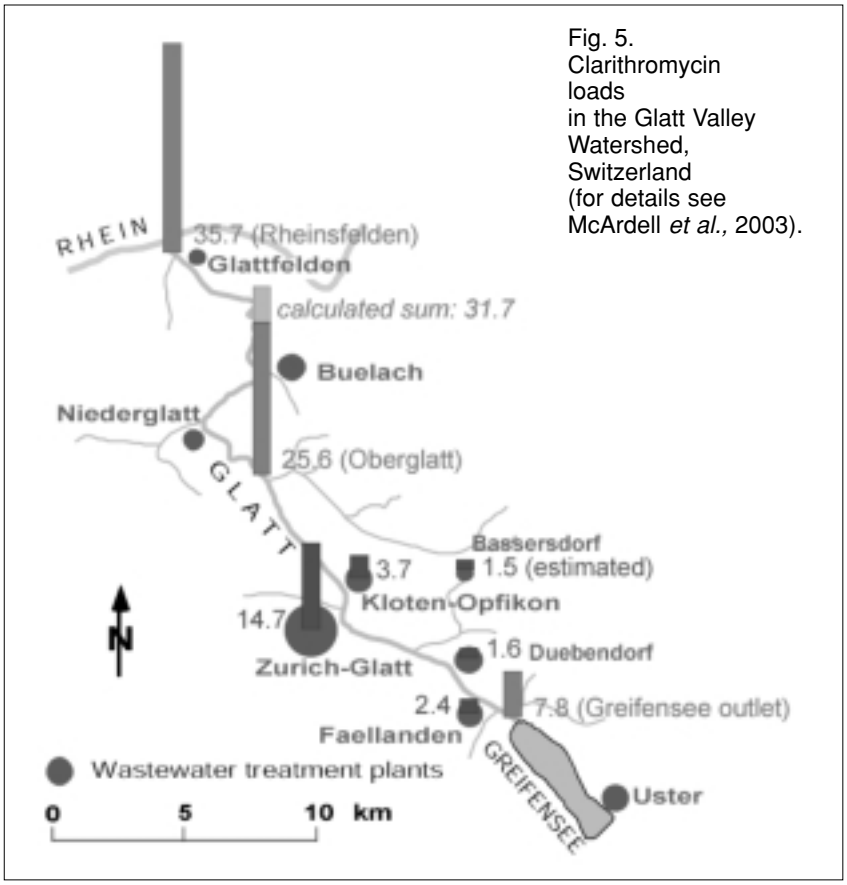

Fig. 5. Clarithromycin loads in the Glatt Valley Watershed, Switzerland (for details see McArdell *et al.,* 2003).

However, such a risk characterization is limited to one compound. Since FQs are very much structure-related, as well as activity-related; the total FQ concentration should be considered in order to account for potential additive toxicity of FQs. For a more advanced and more sophisticated risk characterization, data on mixture toxicities as well on sub-inhibitory effects would be needed.

MACROLIDES

The analytical method for macrolide antibiotics developed by Hirsch *et al*. (1999), which uses LC/MS/MS for analysis, was adapted for analyzing environmental samples by LC/MS (McArdell *et al*., 2003). Due to the fact that no tandem mass spectrometer was available at our institute when this project was started, we had to restrict our investigations to biologically treated wastewater effluents. Interfering peaks in the chromatograms make it necessary to use an approach based on LC coupled to a tandem MS system for analyses of nontreated, or only mechanically treated wastewater samples. We found the following macrolide concentrations in secondary WWTP effluents (i.e. mechanically and biologically treated wastewaters): 57 to 328 ng/l clarithromycin, nondetectable to 287 ng/l erythromycin-H20 and nondetectable to 72 ng/l roxithromycin.

Analyses of water samples from the Glatt river contained clarithromycin concentrations from 7 to 75 ng/l. Using flow rate data of the Glatt river, we could calculate clarithromycin loads along a river stretch of 12 km starting at the Greifensee outlet. The clarithromycin loads increased from 7.8 to 25.6 g/d. The inputs of all WWTPs between these two sampling points were measured yielding a total additional load of 23.9 g/d. The derived removal of 20% is not considered statistically significant because of the uncertainties involved with the chemical determination of trace contaminants and with measuring river flow rates. No clarithromycin elimination was observed in the lower part of the river. These results contrast with the corresponding measurements of the fluoroquinolone antibiotics ciprofloxacin and norfloxacin in the same watershed (Golet *et al*., 2003). Significantly lower concentrations and loads of ciprofloxacin and norfloxacin (max. 10 g/day in winter) and substantial eliminations were observed in the Glatt river.

We can infer from our results that macrolide antibiotics are not fully eliminated in WWTPs and that therefore, residual amounts occur in the receiving surface waters, where macrolide antibiotics are not substantially removed. The goal of decreasing the antibiotics levels in ambient waters can only be achieved by reducing inputs from WWTPs.

Environmental risk management for pharmaceutical compounds as novel contaminants

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INTRODUCTION

Pharmaceuticals, including, human, veterinary, and even illicit (recreational) drugs are a highly variable group of organic compounds with the potential to cause harm to aquatic ecosystems and human health. Thousands of tones of pharmacologically active substances are used annually but surprisingly little is known about their ultimate fate in the environment. A large proportion of an administered dose may be excreted unchanged, while metabolites can be converted back to the active compound via bacterial action (Jones *et al*., 2001). In addition many unused medicines are disposed into the sewage system. A large proportion of urban sewage is contaminated with drug compounds that differ only in their type and abundance. Recent studies have demonstrated that there is incomplete elimination of many pharmaceuticals during sewage treatment and concentration levels from high ng to low μg per litre have been found in surface water, groundwater and marine systems (Ayscough *et al*., 2000). Drugs disposed can end up in landfills, again posing a threat to surface and groundwater (Ahel *et al*., 1998). Moreover, in contrast to more regulated pollutants, which often have longer environmental half lives, the continual introduction of drugs through sewage effluents may have unknown consequences for those aquatic organisms subject to continuous exposure (Daughton and Ternes, 1999). Drugs are used to affect biochemical processes in humans so that environmental exposure of other species may induce on them adverse or even fatal effects (Rand, 1995). In addition, the use of pharmaceuticals is expected to increase following the completion of the human genome project and the increasing age of the population. The data collected to date, however, rarely provides information on the processes that determine their environmental fate. Although they receive considerable pharmacological and clinical testing during development, knowledge of their ecotoxicity is poor. One major concern is that antibiotics found in sewage effluent may cause increased resistance amongst natural bacterial populations (Willis, 2000). As a result, guidelines for new pharmaceuticals have been introduced in the USA and a draft on the environmental risk assessment of new pharmaceuticals is proposed for the EU. The issue is further complicated by the fact that mixtures of only a few compounds have been shown to affect ecosystems in laboratory scale studies. Most organisms are continually exposed to a range of substances with only slight temporal and spatial variations in concentration levels. Consequently, their tolerance will depend on the duration of exposure to many chemical (and non-chemical) stresses, many of which have similar mechanism of action and might behave synergistically. Hence, risk assessments that ignore the possible cumulative effects of pharmaceuticals are likely to lead to a significant underestimation of risks (Jones *et al*., 2002).

RISK ASSESSMENT

The current regulatory approach to the management of synthetic organic chemicals is based almost entirely on human risk assessment, involving: hazard identification - the identification of the inherent capacity of a chemical to cause adverse effects, without regard to the likelihood or severity of such effects; hazard characterisation - the quantitative evaluation of the nature of adverse effects, including assessment of toxic potency (the relative toxicity of a chemical) and, where possible, a dose-response assessment; exposure assessment - the quantitative evaluation of the likely exposure of the environment and, via the environment, of humans to a chemical to evaluate exposure to the chemical of concern; and risk characterisation - the quantitative estimation of the probability that an adverse effect will occur, and of its severity and duration in a given population under defined exposure conditions, based on the three previous elements.

Risk assessment is also used for screening of new chemicals for both the aquatic and terrestrial environment, usually involving calculation of the predicted exposure concentration (PEC) and the predicted no effect concentration (PNEC) and their ratio (PEC/ PNEC). It is performed on the basis of toxicity data for aquatic or soil organisms, or alternatively for higher animals, including humans (Bound and Voulvoulis, 2004). Regulatory approaches based on risk assessment have seriously failed to anticipate some of the deleterious effects of chemicals. For example, the impact of organochlorine pesticides on birds (Hickey and Anderson, 1968; Edwards, 1973; Ratcliffe, 1980; Newton and Haas, 1984) and mammal populations (Chanin and Jeffries, 1978), the adverse effects of PCBs on the health of humans and ecosystems (Stringer and Johnston, 2001; Harrington and Macdonald, 2002), the uptake and bioaccumulation of polybrominated diphenyl ethers (PBDEs) and the endocrine disrupting effects of tributyltin (TBT) and many other persistent organic pollutants (POPs), were not predicted. This approach fails to consider the complexity of the supply chain in which chemicals are used, and fails to incorporate public values including the precautionary principle. Moreover, very few data are available on the distribution of most of the 30,000 synthetic industrial chemicals which include many POPs estimated to be on the market in Europe. In addition, due to the cycling of these compounds between environmental compartments, it can be difficult to distinguish between sources and sinks (Vallack *et al*., 1998).

RISK MANAGEMENT

The need for managing environmental risks associated pharmaceuticals as environmental pollutants is currently attracting a lot of attention. Although more research is needed to elucidate the full scope of potential adverse implications resulting from the occurrence of pharmaceuticals in aquatic environments, there might be scope to take mitigating action and opportunities for prevention. The Precautionary Principle has provided the foundations for building a new risk regulatory pattern under scientific uncertainty (Grandjean *et al*., 2004; Burger, 2003). The management of possible risks can still be effective and useful, even if the uncertainty associated with the risk assessment process is high (Gochfeld, 2003).

The new approach to risk management is through an integrated framework, where there is a better balance between precaution and reality. Although the need for scientific evidence is detrimental to limiting uncertainty, there might still be scope for mitigation measures when for example the benefits associated with those measures outweigh the costs involved. The scientific framework for the management of pharmaceuticals is based on an environmental risk-benefit analysis tool, which uses the conceptual model of 'sources - pathways – impacts' for the presence of chemicals in the environment (Figure 1). Following this, sources, pathways and impacts can be managed in order for risks to be reduced.

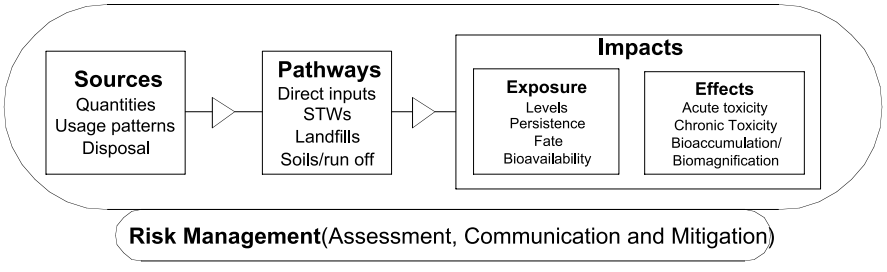

Fig. 1.
Risk management framework for pharmaceuticals in the environment.

Sources:

Around the world thousands of tons of pharmacologically active substances are used annually. A large proportion of an administered dose may be excreted, unchanged while metabolites can be converted back to the active compound via bacterial action, and thus ending up in sewage treatment plants. Disposal of out of date or unwanted medicines may occur via the sink/toilet or in household waste, which is then incinerated or taken to landfill sites.

For example, records of drug use in the UK are kept by the Department of Health (prescribed drugs) and the Proprietary Association of Great Britain (over the counter medicines), and details of the twenty-five most used prescription pharmaceuticals in England, by weight were obtained from the statistics division (SD1E) of the UK Department of Health. Data from 2000 prescription items dispensed by community pharmacists, appliance contractors, dispensing doctors and prescriptions submitted by doctors for items for personal administration are sown in Table 1 (Jones *et al*., 2002).

Table 1. The 25 most used pharmaceuticals by weight in England in 2000.

Compound Name	Therapeutic Use	Total prescription items dispensed (thousands)	Total prescription items where DDD information held (thousands)	Coverage	**Amount used per year (kg)**
Paracetamol	Analgesic	10,636	10,636	100%	390954.26
Metformin hydrochloride	Anti-hyperglycaemic	3,596	3,596	100%	205795.00
Ibuprofen	Analgesic	6,683	5,422	81%	162209.06
Amoxycillin	Antibiotic	12,849	12,849	100%	71466.83
Sodium valproate	Anti-epileptic	1,495	1,495	100%	47479.65
Sulphasalazine	Anti-rheumatic	622	622	100%	46430.43
Mesalazine (systemic)	Treatment of ulcerative colitis	622	622	100%	40421.72
Carbamazepine	Anti-epileptic	2,256	2,256	100%	40348.75
Ferrous sulphate	Iron supplement	2,639	2,639	100%	37538.52
Ranitidine hydrochloride	Anti-ulcer drug	3,770	3,770	100%	36319.24
Cimetidine	H_2 receptor antagonist	1,496	1,496	100%	35654.20
Naproxen	Anti-inflammatory	1,381	1,335	97%	35065.98
Atenolol	_-blocker	11,554	11,554	100%	28976.55
Oxytetracycline	Antibiotic	1,195	1,195	100%	27195.11
Erythromycin	Antibiotic	2,936	2,573	88%	26483.78
Diclofenac sodium	Anti-inflammatory & Analgesic	7,639	7,134	93%	26120.53
Flucloxacillin sodium	Antibiotic	2,552	2,552	100%	23381.47
Phenoxymethylpenicillin	Antibiotic	2,716	2,716	100%	22227.59
Allopurinol	Anti gout drug	2,038	2,038	100%	22095.64
Diltiazem hydrochloride	Calcium antagonist	2,844	2,844	100%	21791.50
Gliclazide	Anti-hyperglycaemic	3,060	3,060	100%	18783.11
Aspirin	Analgesic	16,769	1,305	8%	18105.89
Quinine sulphate	Muscle relaxant	1,633	1,633	100%	16731.26
Mebeverine hydrochloride	Anti-spasmodic	1,323	1,323	100%	15497.35
Mefenamic acid	Anti-inflammatory	544	544	100%	14522.77

a: Figures relate to the Health Authority where the prescription was dispensed not where it was prescribed.

b: The weight of the chemical dispensed is based on Defined Daily Dose (DDD) information. Note that the DDD data do not cover all individual drugs. The "coverage" column indicates for each chemical the percentage of the prescriptions dispensed where DDD data are held. For example the weight of aspirin dispensed is based on only 8% of prescription items dispensed.

Pathways:

Most human pharmaceuticals are released by excretion from the patient or, to a lesser extent, in aqueous waste produced in manufacturing. Sewage treatment plants (STPs) may therefore be assumed as the focal point of collection and subsequent release into the environment (Figure 2). In addition, disposal of waste medicines by the general public is often considerable. When a course of medication is prescribed, there is often strong emphasis on the need to finish it completely. This is not only important on medical grounds, but it also reduces the chance for any unfinished prescriptions (Ares, 1999). If a patient should have any left over medicines the correct procedure is (in Europe at least) to return them to the pharmacy, which is then responsible for disposal through the use of special, licensed waste disposal contractors. In practice, this is often not the case (Slack *et al*., 2004). The public is under no obligation to return unused or expired medicines to pharmacists for safe disposal; such action is often dependent on whether clear advice is given, for example, in accompanying patient information leaflet. This guidlines do not apply in America (Daughton and Ternes, 1999). Realistically, the majority of people will either flush unused drugs down the drain (where they will eventually pass to a STP) or dispose of them in domestic refuse which will ultimately enter domestic waste landfill sites or, to a lesser extent, be incinerated. Both of these routes represent a risk to the environment (Ternes, 2000).

The majority of compounds are unlikely to be degraded in a STP and most are unlikely to sorb to sludge; therefore, these substances could be discharged to rivers. This leads to serious consequences in certain parts of the UK, where water is supplied through direct abstractions. Consequently, a large proportion of the flow during dry periods may be made up of treated sewage effluent. Such a high rate of abstraction and reuse means there is a possibility that pharmaceuticals and/or their metabolites might enter the public water supply.

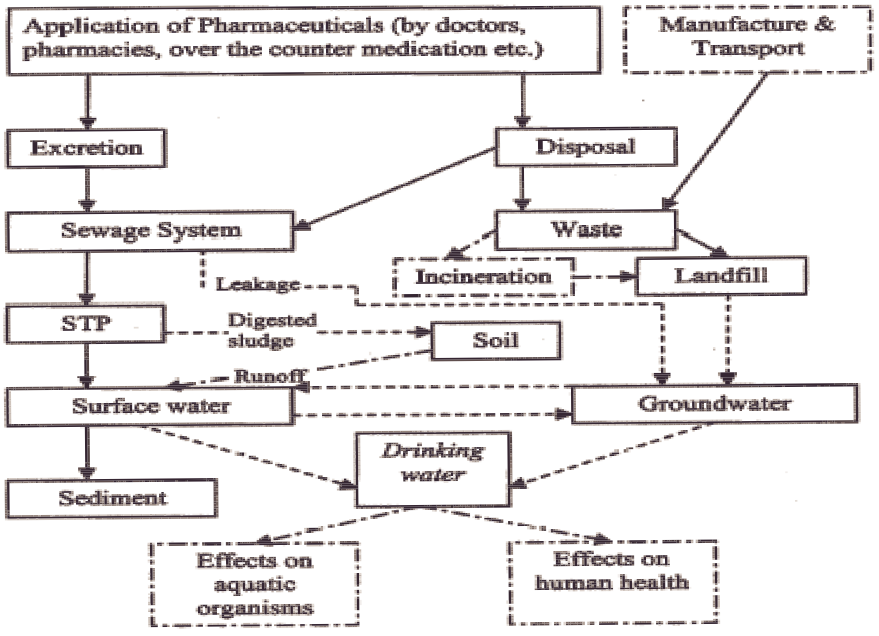

Figure 2.Possible pathways of human pharmaceuticals in the environment.
(Key to figure, ——— strong evidence, - - - - - medium evidence, —·—·— unknown/weak evidence).

Fig. 2. Pathways of human pharmaceuticals in the environment.

Impacts:

There are numerous concerns regarding the hazards of pharmaceutical compounds in the environment, and it is frequently recommended that more research should be done in this area. Overall, a more diverse range of animal species with defined genders and physiological status should be tested. However, to avoid animal testing, in vitro tests with detailed correlation to in vivo tests should be undertaken. This is of benefit, as it should help provide an understanding of the mechanisms involved in the responses observed. Moreover, properties such as exposure

routes, timing, frequency and duration, and the presence and absence of other possible contaminants are necessary for detailed dose-response assessments.

One area of particular interest is the potential for pharmaceuticals to re-enter the human body. When surface waters are used as sources of drinking water, abstraction points may often be down-stream of effluent discharge points, and groundwater sources have also been found to be contaminated with pharmaceutical compounds (Sacher *et al*., 2001). In densely populated urban areas with high municipal wastewater discharges and low surface-water flows there is a potential risk of drinking-water contamination by polar organic compounds (Froese *et al*., 1999).

It is difficult to extrapolate laboratory-based acute toxicity data to the lower concentrations and routes of exposure encountered in the environment. There remains a wide range of issues relating to the occurrence of potential effects that requires further investigation before the environmental significance of this problem can be fully evaluated. Although advances in analytical chemistry have driven this area of research (pharmaceutical pollution at these levels was not routinely detectable even 10 yr ago and hence was not considered a threat), the development of analytical methods is still an essential part of improving uncertainty, and methods for the determination of drugs in solid phases such as sediments would also be useful (Jones *et al*., 2004).

It is unlikely that pharmaceutical compounds are present in the environment at concentrations high enough to cause significant harm. However, at sufficient concentrations they have been observed to induce effects in both animals and some plants, and it is possible they may have other effects that have not yet been observed. It would be unwise to assume these compounds ineffective until there is conclusive proof (Jones *et al*., 2003).

CONCLUSIONS

The widespread dispersion and high usage volume of pharmaceuticals will most likely lead to a more or less constant presence, albeit in low concentrations, in rivers and other water-bodies. In addition, these compounds will have chronic, rather than acute toxic effects, for example by causing a change in behaviour that reduces the individual fitness of an organism. Poorly characterised processes warrant a more precautionary view on possible environmental fate and effects. Available scientific knowledge is less than that needed to fully assess the risks these compounds pose to the environment, and future work will need to focus on more detailed assessments of specific pathways and effects in the aquatic environment.

Due to their beneficial health effects and economic importance, the best available evidence and data will be required to fully evaluate the costs and benefits before any actions are taken to reduce inputs of drugs to the environment. Pollution control efforts could focus more on reduction, minimization, and elimination at source, where possible, while other policies could include the development of clearer labelling on medicinal products and better guidelines for the disposal of pharmaceutical compounds by patients and medical professionals. This approach would have the potential benefit of improved consumer health (by minimizing the intake of active substances), as well as reduced health care spending.

Sewage treatment works are likely to be the most significant source of human medicinal compounds to surface waters, while the application of sewage sludge on agricultural land may also contribute a high load of drugs to the aqueous phase after runoff events. Sewage is a continuous point source, while runoff from agriculture is diffuse, where concentrations are dependent on the application rate and runoff parameters.

Laboratory data on the toxicity of compounds gathered during product development may be able to provide useful information for risk management. The medical and social value of drugs is unquestioned, but more data are needed about the ecotoxicological effects of medicines. There will also be a need to adjust the assessment to the specific environmental compartment, organism, and endpoint of the drug in question.

Current and future environmental risk assessment of pharmaceuticals

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INTRODUCTION

Recent years have seen an increasingly detailed view on the occurrence and effects of human and veterinary pharmaceuticals in aquatic ecosystems (Ternes, 1998; Kolpin *et al*., 2002; Heberer, 2002). The old "cradle to grave" paradigm that ended in the patient's body has to be adapted and must be enlarged by environmental fate and effect considerations. It has become evident that pharmaceuticals represent a novel class of substances consistently present in the environment at trace levels. Regulatory approaches have been taking this aspect into account, typically since the early 90's. Various environmental risk assessment procedures exist for human and veterinary pharmaceuticals (EMEA, 2000; EMEA, 2001; FDA, 1998). For the latter, an internationally harmonised VICH guideline is in place for Tier 1. For human pharmaceuticals, the US, EU and Canada require specific risk assessments from manufacturers submitting marketing applications (reviewed by Straub, 2002).

The basic approaches used in these assessments are not completely different from chemical environmental risk assessment: tiered decision trees with a stepwise increasing level of detail and data needs have been set up. As a prioritisation tool, *de minimis* or "threshold of concern" exposure levels are used prior to entering Tier 1, a tonnage or use based worst case exposure estimation, refinable by various depletion mechanisms such as metabolism or biodegradation, effect assessment based on acute effect data in three species. A next higher tier with a refined fate or effect assessment has to be entered if risk ratio in an earlier tier indicates a concern (e.g. PEC/PNEC >1). Details can be taken from the original guidelines, which are all publicly available, partly even online (see Guidelines in the references).

In general terms, pharmaceuticals differ from industrial chemicals in several aspects:
- they are designed for interactions with receptors;
- they are sometimes highly active, and most often highly specific;
- high activity often results in relatively low tonnage;
- extensive knowledge exists on e.g.: *in vitro*/*in vivo* mode of action, target receptors, mammalian toxicity;
- unique physicochemical properties: many are non-neutral, ionizable or amphiphilic;
- biodegradability is not a priority in current drug design (see below).

The uniqueness of pharmaceuticals compared to other environmental contaminants supports why no other class of compounds has been tested to a similar extent in biological systems (mostly mammalian, sometimes others). By taking pharmaceuticals through time and cost intensive *in*

silico, *in vitro* and *in vivo* (preclinical and clinical) testings, many unwanted toxicological and pharmacological side effects can be excluded by the time a drug reaches the market. Even interactions with other drugs (DDI= drug-drug interactions), at least at pharmacologically relevant concentrations, are being assessed during ADME/Tox screening activities. Out of these considerations, it seems self-evident that a sensible environmental risk assessment of pharmaceuticals must take these data into account.

Ultimately, the medical benefits of pharmaceuticals have to be taken into account and weighed against any possible detrimental effect on ecosytems. The goal should be an optimal availability of pharmaceuticals to the global community and equal concern for health to the global ecosystem.

EXPOSURE OF PHARMACEUTICALS TO MARINE ECOSYSTEMS

At first glance, human pharmaceuticals might seem as complete strangers to marine ecosystems, yet they're not. Many, especially highly active pharmaceuticals, are not new to the marine environment, but have been isolated originally from marine species, e.g. antiviral compounds, antibacterials and anticancer drugs. Table 1 gives a few examples.

Table 1. Pharmaceuticals isolated from marine organisms that are currently marketed or under development.

Compound	Therapeutic purpose	Isolated from	Mechanism
Aurantoside B	Antifungal	*Siliquariaspongia japonica* (sponge)	Inhibition of tubulin polymerization
Cribrostatin 3	Antibacterial	*Cribrochalina* sp (blue sponge)	Not yet known
Squalamine	Anticancer	*Squalus acanthias* (dogfish shark)	Neovascularization inhibitor
Kalihinol F	Anticancer	*Acanthella* sp, (sponge)	Topoisomerase I inhibitor
Arabinosyl nucleosides	Antiviral (precursors for Ara-C, Ara-A,…)	*Cryptotethia crypta* (sponge)	Inhibitor of viral reverse transcriptase
Nostodione A	Anticancer	*Nostoc commune* (blue green algae)	Mitotic spindle poison
Bryostatin-1	Anticancer	*Bugula neritina* (bryozoans)	Protein Kinase C inhibitor

Sources: Donia and Hamann, 2003; Haefner, 2003; Sills *et al.*, 1998; Ohta *et al.*, 2003.

This should by no means be an argument to allow exposure of the environment to uncontrolled loads of pharmaceuticals. It is obvious that these compounds are naturally restrained to the specific organisms that developed them as a competitive advantage throughout evolution. However, it supports the thinking that the data generated during drug optimization for molecular targets in mammalian systems (receptors, enzymes, structural proteins, DNA etc.) might be a useful start to look at possible effects of the same compounds, after their therapeutic application, in other, non-mammalian biological systems.

GENERAL EXPOSURE SITUATION

From both available tonnage information (prescription or production) as well from the measured environmental concentrations, it is emerging that pharmaceuticals are a new class of anthropogenic contaminants of year-round occurrence and at generally low levels in the environment. For high-volume pharmaceuticals, loads can be assumed comparable to those of some pesticides (BLAC, 2003).

Pharmaceuticals reach the aquatic environment via different routes after their application in target organisms. Human pharmaceuticals are primarily spread via municipal sewage systems and sewage treatment plants, provided they are neither mineralized nor adsorbed to activated sludge. In the latter case, they can infiltrate fields and, depending on their mobility, reach groundwater systems or even well water.

For veterinary pharmaceuticals, the main pathway relevant for aquatic systems follows the line manure → soil → ground water. Via surface runoff, direct entry of veterinary pharmaceuticals into rivers and lakes is also possible.

Up to now, most data on exposure has been generated in wastewater and freshwater systems. The general picture shows that hardly any environmental matrix, including seawater, groundwater and drinking water, is devoid of low (ug/L) or very low (pg-ng/L) levels of pharmaceuticals. In general, concentrations decrease considerably along the distribution pathway, i.e. from untreated sewage to sewage effluent, surface waters (rivers > lakes), seawater, groundwater and drinking water. Analytical data from marine environments are still scarce, but the available data show that levels are generally in the low ng/L range, with somewhat higher values in the plumes of major rivers (Buser, 1998; Weigel, 2002; Weigel, 2001).

Human Health Risks

Currently, there is no data to indicate that there is a human health risk posed by the levels present in drinking water and through the consumption of aquatic organisms, mainly fish (Schulman *et al*., 2002; Webb *et al*., 2003). Given the arguably low doses ingested even by lifelong exposure compared to therapeutic doses, this is likely to hold true for both the general population as well as for potentially more susceptible subgroups such as pregnant women, children or health compromised subjects. Industry sponsored activities in this area will help to further clarify this issue.

Data on ecological effects of pharmaceuticals

There is currently no concern that acute effects on aquatic systems must be expected. Exceptions can occur locally when untreated production or municipal waste is shed directly into surface water or estuaries. However, emerging contaminants such as pharmaceuticals are unlikely to be the primary damage causing agents in these situations. Primary pollutants such as ammonia, heavy metals and other anthropogenic, industrial or agricultural toxins define ecosystem health ("dead fish do not care for endocrine disruption"). There is, however, some scientific uncertainty regarding the environmental impact of pharmaceuticals due to their specific modes of action, the chronic exposure situation of aquatic life and the complex mixture situation (Seiler, 2002).

Ecological effect data taking into account chronic exposure and mixture effects are being increasingly published (Cleuvers, 2004; Ferrari *et al*., 2003).

Environmental Risk Assessment

The current risk assessment procedures do not specifically take into account maritime systems, but rely on established sentinel species, mostly freshwater organisms, which may not be representative of all organisms potentially exposed to pharmaceuticals in coastal waters. However, a recent sensitivity comparison for freshwater vs. saltwater species showed a generally small difference in sensitivity to environmental contaminants compiled in the EAT3 database (ECETOC, 2003). 78% of all data were not more than tenfold different in sensitivity of fresh- vs. saltwater species – in either direction. For chronic NOECs, there was even a slight trend towards freshwater species being more susceptible to the EAT3 chemicals. Nevertheless, it appears that our knowledge on the susceptibility of certain key marine taxa such as Echinodermata, Mollusca, Cephalopoda or Ctenophora towards pharmaceuticals is not yet advanced enough to judge whether marine ecosystems are sufficiently protected by the current environmental risk assessments.

The present environmental risk assessments have generally not indicated adverse effects on ecosystems. In the very few cases where a concern surfaced, appropriate risk reduction measures were taken to minimize environmental impacts (e.g. restriction of application to hospitals, restricted access to surface water for treated animals etc.). Recently, a debate has started on whether current ERA procedures underestimate the ecological impact of pharmaceuticals, due to their focus on acute effect data and the use of safety factors derived from non-specifically acting chemicals (e.g the EU TGD proposals). Typically, a safety or assessment factor of 1000 is applied to the lowest available acute LC50 or EC50 from fish, daphnia or algae. By applying these factors, an acute to chronic ratio (ACR) of 10-100 is assumed, which has indeed been found for

a large number of industrial chemicals (Lit). Some researchers now claim that for specific pharmaceutical classes, ACRs of 10,000 or higher might be appropriate for ERA. While research in this field is ongoing, it might be useful to take a closer look at an interesting dataset recently published by ECETOC: 38 specifically acting substances out of the EAT3 database showed a median ACR of 10.6, with a minimum -maximum range of 1.33 – 34800 and a 95th percentile ACR of 191 (ECETOC, 2003). Published chronic data on pharmaceuticals are scarce. A few available examples have been compiled below and the respective ACRs have been calculated.

Table 2. Acute to Chronic Ratios (ACRs) in vertebrates for a selection of human pharmaceuticals.

Substance	**Acute data**		**Chronic data**		**ACR**	**Ref. (chronic data)**
	Type	Value	Type	Value		
Ethinylestradiol	LC_{50}	1.5 mg/L	Chronic NOEC	1 ng/L	1.5 *10^6	Laenge *et al.*, 2001
Estradiol	LC_{50}	6.1 mg/L	Chronic EC_{50}	0.12 µg/L	50'833	Kramer *et al.*, 1998
Propranolol	LC_{50}	24.3 mg/L	Chronic LOEC	0.5 µg/L	48'600	Huggett *et al.*, 2002
Fadrozol	LC_{50}	49 mg/L	Chronic LOEC	2 ug/L	18'500	Ankley *et al.*, 2002
Carbamazepine	LC_{50}	43 mg/L	Chronic NOEC	25 µg/L	1720	Ferrari *et al.*, 2003
Diclofenac	LC_{50}	50 mg/L	Chronic NOEC	1 mg/L	50	Ferrari *et al.*, 2003
Thiabendazole	LC_{50}	0.56 mg/L	Chronic NOEC	0.012 mg/L	46	EPA online data [a]

[a] http://www.epa.gov/pesticides/reregistration/thiabendazole/

This compilation indicates that current risk assessment schemes with their typical assumption of acute to chronic ratios in the range of 10-100 might be underprotective for some specific classes of pharmaceuticals. On the other hand, numerous pharmaceuticals are known to have ACRs comparable to, or even below those of industrial chemicals, like e.g. the X-ray contrast agent iopromide with an ACR of 1 (Steger-Hartmann *et al*., 2002).

NEW CONCEPTS FOR THE FUTURE?

There is increasing agreement on the concept that a sensible ERA for pharmaceuticals has to take into account their specific mode of action and should leverage the huge biological and toxicological knowledge that accumulates during drug development (Laenge and Dietrich, 2002).

A possible concept of how the first step of a pharmaceutical portfolio screening is shown in Figure 1.

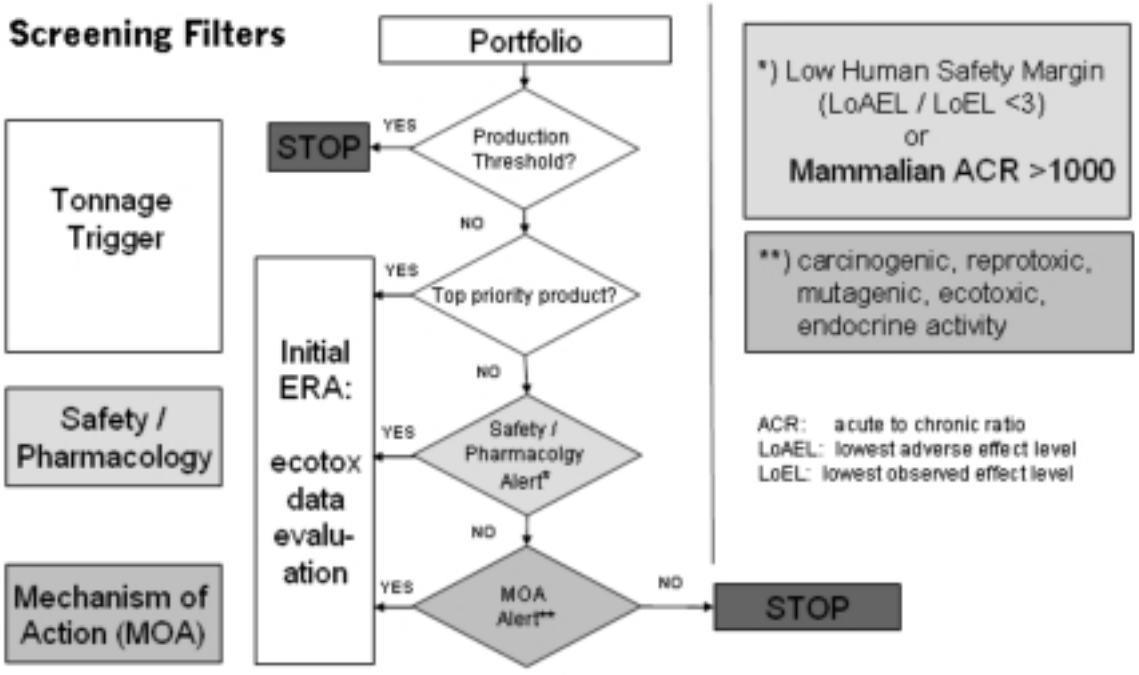

Fig. 1. Possible mechanism and toxicity based decision criteria for environmental risk prioritization.

For new pharmaceuticals, the development of a "green chemistry" concept has been suggested, aiming at yielding "environmentally optimized" drugs that are safe, have a high activity and efficacy, but are cleared rapidly from the environment after their excretion and release into aquatic systems (Daughton, 2003). While this approach is a valuable aim, there are still significant obstacles that need to be overcome in order to guarantee the availability of innovative drugs for patients, while assuring global ecosystem wellbeing.

Besides complete mineralization or unchanged sorption to sediments, sludge or soil, two key mechanisms can lead to a depletion or inactivation of pharmaceuticals during and after their use in patients: metabolic transformation and photochemical degradation. Metabolism is indeed a very important mechanism that can inhibit accumulation in body tissue or prevent unnecessarily slow removal (clearance) of a pharmaceutical from the body. On the other hand, metabolically unstable drugs have been responsible for a number of very serious side effects that have even led to market withdrawal due to safety issues (Jaeschke *et al*., 2002). It is a fact that the mechanistic cause of most drug related cases of idiosyncratic hepatotoxicity in man is caused by reactive metabolites generated by oxidative CYP450 metabolism. These metabolites can cause direct toxic effects by interacting with biological nucleophiles (e.g. amino groups or sulfhydryl groups contained in glutathione, proteins or DNA). Alternatively, they can trigger an immunotoxic event by covalently binding to its metabolizing enzyme and changing its three-dimensional shape. This altered protein-hapten complex is then recognized as non-self and an immunological response follows.

Medical radioisotopes entering the marine environment

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A number of radioisotopes are used extensively in hospitals for both diagnostic and therapeutic treatments. There is concern and considerable ignorance regarding the release and impacts of radioactivity from medical applications, including ultimate discharge into coastal waters through the effluents of sewage treatment plants. Here we consider this issue and assess the fate of discharged radioactivity associated with medical use into coastal ecosystems.

Table 1 presents a list of commonly used radioisotopes in medical practice. In the case of diagnostic medical testing, the patient and his excreta are radioactive until the material has decayed or been excreted. ^{99M}Tc, ^{67}Ga, ^{111}In and ^{201}Tl are among the most commonly used isotopes in this capacity. Therapeutic treatments use both external and internal radiation sources. The use of external radiation, known as teletherapy, does not render the patient radioactive, whereas in brachytherapy, where internal radiation sources are placed inside the body, the patient becomes radioactive. Sealed sources used in brachytherapy do not render the patient's excreta radioactive, but unsealed brachytherapy sources are metabolized by the patient, rendering his wastes radioactive. ^{32}P, ^{89}Sr and ^{131}I are among the most commonly used isotopes for unsealed sources. It is of course critical to consider the radioactive half-lives of the radioisotopes that might be released from the patient; if these are short relative to geochemical and biological processes that result in their bioaccumulation and potential toxicity, then there may be no cause for concern with respect to deleterious effects on organisms in receiving waters. Radioactive excreta are usually disposed of in the sewer system. In coastal communities, sewage effluent is commonly released into estuaries, harbors, and other coastal waters.

Generally, there have been few studies documenting the behavior of radioactive materials in municipal sewage (Ainsworth *et al*., 1994; Martin and Fenner, 1997), even though there has been an increase in the use of radioisotopes in medicine in the last 30 years. Most have focused on the concentrations and behavior of ^{131}I in municipal sewage and sewage sludge; relatively little is known about the occurrence and behavior of other short-lived radioisotopes used in medicine that may enter the sewer system via patient waste. Table 2 summarizes eleven studies and the maximum concentrations of ^{131}I measured at various sewage treatment plants. Here we discuss an example of the medical application of ^{131}I and its eventual release through the sewer system into coastal waters. ^{131}I is one of the most widely used medical radioisotopes and is commonly used in the treatment of thyroid cancer and hyperthyroidism. The total radioactivity of sewage from hospitals is often dominated by this one isotope. The example we consider, based on a local municipality in suburban New York, is probably representative of numerous similar situations in urban and suburban settings throughout N. America and Europe. The findings are presented to illustrate the likely fate of discharged radioisotopes into coastal waters.

Table 1. Commonly used radioisotopes in nuclear medicine and radiation oncology, including their principal types of radioactive emissions, radioactive half-lives, and whether they can be released into sewage systems following typical medical applications.

Radioisotope	Principal Use	Principal Type of Emission	Radioactive Half-life	Discharged into Sewer (yes/no)
^{67}Ga	diagnostic	gamma	3.3 d	yes
^{99m}Tc	diagnostic	gamma	6 h	yes
^{111}In	diagnostic	gamma	2.8 d	yes
^{201}Tl	diagnostic	gamma	3 d	yes
^{123}I	diagnostic	gamma	13 h	yes
^{131}I	diagnostic and unsealed therapeutic	beta, gamma	8 d	yes
^{32}P	unsealed therapeutic	beta	14 d	yes
^{89}Sr	unsealed therapeutic	beta	51 d	yes
^{198}Au	sealed and unsealed therapeutic	beta, gamma	2.7 d	yes
^{137}Cs	sealed therapeutic	beta, gamma	30 y	no
^{133}Ba	sealed therapeutic	gamma	10.5 y	no
^{60}Co	sealed therapeutic	beta, gamma	5.3 y	no
^{192}Ir	sealed therapeutic	beta, gamma	74 d	no
^{125}I	sealed therapeutic	gamma	60 d	no
^{90}Sr	sealed therapeutic	beta	29 y	no

^{131}I emits both ß$^{-}$ particles and γ rays. Driver and Packer (2001) found that 55% of the initial activity of ^{131}I given to patients treated for thyroid cancer is excreted in the first 24 hours after treatment and 85% is excreted over a five-day period. Although the isotope is short-lived, medical patient waste represents a constant source of ^{131}I to the sewer system. Generally, 1-20% of ^{131}I that enters sewage treatment plants is typically found in the sludge, with most of the remainder leaving in the effluent (Prichard *et al*., 1981; Martin and Fenner 1997; Puhakainen, 1998). Rose (2003) measured ^{131}I concentrations at three sewage treatment plants on Long Island, New York. The highest concentrations (148 ± 4 Bq g^{-1} dry sludge and 63 ± 2 Bq L^{-1} effluent) were found at a plant that serves the Stony Brook University Hospital. This hospital is relatively large, well equipped and staffed for thyroid cancer treatments, for which it treats approximately 70 inpatients per year. The patients generally receive an oral dose (5.55 to 6.48 GBq) of ^{131}I and remain in the hospital for 30 to 36 hours following treatment. Urine and feces are released into the sewer system and not held. The hospital also treats about 45 hyperthyroidism cases (0.37 to 1.11 GBq of ^{131}I each) and performs about 150 whole body scans (0.19 to 0.22 GBq of ^{131}I each) each year. These patients do not remain in the hospital. Patients treated for thyroid cancer remain in the hospital and receive the highest doses of ^{131}I, so the variation in the Stony Brook sewage treatment plant is primarily a function of these treatments.

Table 2. Summary of previous studies and the highest ^{131}I concentrations measured at several sewage treatment plants. Note: 1 Bq = 1 disintegration second^{-1}.

Investigation	Sewage Treatment Facilities Investigated	Maximum ^{131}I Concentrations Reported	Product Analyzed
Prichard *et al.*, 1981	10 in 9 U.S. cities	0.025 Bq g^{-1}	wet sludge
Erlandsson and Mattsson, 1978[1]	Malmö Sweden	0.004 Bq g^{-1}	digested sludge
Durham and Joshi, 1979	Hamilton, Western Lake Ontario, Canada	0.002 Bq g^{-1}	dry sludge
	Dundas, Western Lake Ontario, Canada	0.007 Bq g^{-1}	dry sludge
Larsen *et al.*, 1992[2]	Oak Ridge, Tennessee	0.006 Bq g^{-1}	liquid digested sludge
Barci-Funel *et al.*, 1993[1]	Nice, France	0.055 Bq g^{-1}	dewatered sludge
	St. Laurent du Var, France	0.027 Bq g^{-1}	dewatered sludge
Dalmasso *et al.*, 1997[1]	Nice, France	0.26 Bq g^{-1}	dewatered sludge
	St. Laurent du Var, France	0.109 Bq g^{-1}	dewatered sludge
Puhakainen, 1998	4 in Finland	0.25 Bq g^{-1}	sludge
Washington State Dept of Health, 1997	6 in Washington State	1.2 Bq g^{-1}	dry sludge
National Biosolids Partnership, 1999	55 in 17 states	6.5 Bq g^{-1}	sludge cake
Sodd *et al.*, 1975[3]	Millcreek, Cincinnati, Ohio	5.0 Bq L^{-1}	effluent
Martin and Fenner, 1997	Ann Arbor, Michigan	192 Bq L^{-1}	effluent

1. At the time of the study, urine from patients receiving 3.7 GBq or more of ^{131}I was collected and held in the hospital.
2. This value represents an annual mean concentration.
3. At the time of the study, approximately 60% of the patients were treated as outpatients.

Assuming that 70 thyroid cancer cases are treated at the University Hospital each year, that each patient receives 5.55 GBq of ^{131}I and excretes 55% of the dose into the sewer system while in the hospital, the annual discharge from the hospital would be approximately 222 GBq. This exceeds the allowable discharge limit in the United States for other types of radioactive materials. Some hospitals in other countries have installed systems to delay thyroid cancer patient waste before entering the municipal sewer system (Goddard, 1999; Leung and Nikolic, 1998) while others collect urine from patients receiving doses of 3.7 GBq or more of ^{131}I to allow for decay (Erlandsson and Mattsson, 1978; Barci-Funel *et al.*, 1993). Once it enters a sewage treatment facility, up to 95% of the ^{131}I leaves in the effluent, which, in the case of the Stony Brook facility, is discharged into Port Jefferson Harbor, New York. The sewage effluent represents an essentially continuous point source of ^{131}I for the harbor.

Because radioisotopes behave identically with their stable analogs, it is appropriate to consider studies describing the speciation of trace elements in seawater in order to understand the fate of radioisotopes that enter coastal waters. In the case of iodine, iodide (I^-) and iodate (IO_3^-) dominate in aqueous systems (Turner *et al.*, 1981). Iodine generally shows relatively little enrichment in coastal sediments, with mean partition coefficients (Kds) on the order of 10^2 (IAEA, 2004). However, it is known to concentrate in a number of marine organisms, most

particularly macroalgae and plankton (phyto and zooplankton), where concentration factors (degree of enrichment in organism relative to ambient seawater) average 10^3 (Table 3). This element is considerably less enriched in most animals (e.g., fish, mollusks, most crustaceans), for which concentration factors are on the order of 10^1 (Table 3). Consistent with this known behavior, ^{131}I in Port Jefferson Harbor was most detectable in the brown macroalga Fucus sp., with the highest concentrations (0.260 ± 0.005 Bq g^{-}1) measured nearest the sewage outfall. Once it is bioconcentrated by algae, radioiodine can be transferred to animals which consume these organisms, as shown in England, where radioiodine was concentrated in macroalgae and subsequently consumed by swans, where it concentrated in their thyroid glands (Howe and Hunt, 1984). ^{131}I from medical sources can also be measured in rapidly accumulating, recently deposited sediments, such as in New York Harbor (Oktay *et al*., 2003), where it may serve as a source for benthic organisms. The behavior of this radioisotope in benthic ecosystems has not been well studied.

Table 3. Log concentration factors (concentration of element in organism divided by concentration of element in equal mass of ambient seawater) for medical radioisotopes potentially releasable into coastal waters. Data for Tc, In, Tl, I, and Sr from IAEA (2004), for Ga, P, and Au from Lowman *et al.* (1971). nd: no data available; ? denotes considerable uncertainty about stated value.

Element	Fish	Crustaceans	Molluscs	Seaweeds	Phytoplankton	Zooplankton
Ga	nd	3.3	3.3	3.1	3.9	3.8
Tc	1.9	3	2.7	4.5	0.6	2
In	3	4	4?	3.7?	3?	4?
Tl	3.7	3?	3.8	3?	3?	3?
I	1	1.3	1	4	3	3.5
P	4.5	4.4	3.8	4	4.5	4.1
Sr	0.3	0.7	1	2.3	0.5	0.5
Au	1.8	2.6	2.6	2.7	nd	nd

The radioactivity from bioconcentrated ^{131}I can be compared to radioactivity from naturally occurring radionuclides in marine systems. In the case of ^{131}I in seaweeds in Port Jefferson Harbor, the radioactivity was approximately one third of that attributable to naturally occurring ^{40}K and about 7-19 times greater than that of the second-most abundant naturally occurring radioisotope, ^{210}Po (Table 4). Radiotoxicity is a function not just of total radioactivity but of the type of radioactive emissions, where alpha emitting radioisotopes are far more toxic than beta and gamma-emitting radioisotopes. Since the total radioactivity from ^{131}I is lower than that from ^{40}K and the potential damage is greater from the alpha emissions of ^{210}Po in these plants, there is no reason to expect radiotoxicity attributable to the accumulated ^{131}I in these algae. However, animals that consume these algae may, in fact, concentrate the ^{131}I in some organs, particularly the thyroid gland, as noted above for swans, and hence consideration of the trophic transfer of this radioisotope is appropriate for evaluating possible toxicity in coastal and riverine ecosystems for higher animals.

As a general rule, by combining the amounts of radioisotopes employed, their radioactive half-lives and types of emissions, and the observed bioconcentration patterns of their stable analogs in select marine organisms (Table 3), one could identify, in principle, which radioisotopes—if any—have the greatest probability of generating detrimental effects to aquatic organisms or their consumers. We believe that this approach provides a rational means by which risks associated

Table 4. Typical concentrations of several naturally occurring radionuclides in seawater (Bq L^{-1}) or marine sediments (Bq g^{-1}) (Joseph *et al.,* 1971) and in seaweeds (Bq g^{-1}) (Sam *et al.,* 1998). For comparison, the radioactivity attributable to ^{131}I in seaweeds near the sewage outfall in Port Jefferson Harbor, New York is shown.

Radionuclide	Concentration in Seawater (Bq L^{-1})	Concentration in Marine Sediments (Bq g^{-1} dry wt)	Concentration in Seaweeds (Bq g^{-1} dry wt)
^{210}PO	0.003	0.15	$1.4 \times 10^{-2} – 3.6 \times 10^{-2}$
^{226}RA	0.003	0.15	$9.6 \times 10^{-3} – 3.7 \times 10^{-3}$
^{230}TH	<0.0002	0.15	$6.2 \times 10^{-4} – 2.1 \times 10^{-3}$
^{238}U	0.033 – 0.042	0.012	$1.2 \times 10^{-2} – 2.9 \times 10^{-2}$
^{40}K	11	0.2 – 0.94	$7.9 \times 10^{-2} – 8.3 \times 10^{-1}$
^{131}I			2.6×10^{-1}

with radioactivity discharges could be assessed. Again, it is most instructive to put the risks of any discharges into the context of the natural radiation background that all marine organisms, and their consumers, experience. Given the relatively short half-lives (Table 1) of most of the radioisotopes commonly used in medicine for diagnostic or unsealed therapeutic purposes (and thus releasable into coastal waters through sewage treatment facilities), it would appear that the likelihood of detrimental effects is low. However, a steady input of these isotopes into coastal waters, such as often occurs with large medical facilities, may result in sufficient bioaccumulation and trophic transfer in marine food chains which would require investigation. By employing straightforward laboratory and field studies, irrational fears about the dangers of discharged radioactivity could be relieved and proper guidance regarding seafood consumption from different waters, if indeed any precautions are warranted, could be generated.

Emerging pathogens in coastal areas

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INTRODUCTION

On the basis of diseases occurring in the human or animal populations, pathogens might be expected in recreational waters or in shellfish from time to time. Thus, the presence of microorganisms - among then emerging pathogens- such as human enteric viruses (norovirus, astrovirus, rotavirus, hepatitis A virus), pathogenic bacteria (*Salmonella, Listeria monocytogenes,* Shiga-Toxin-Producing *E. coli* (STEC)*, Vibrio cholerae, Vibrio parahaemolyticus*, etc.*)* has been reported in coastal areas. Those microorganisms have been implicated in gastrointestinal and respiratory illnesses and other infections (skin, eyes, etc.), (Griffin *et al.,* 2003; Koopman and Duizer, 2003). Some of them, especially viruses, are responsible for shellfish-borne diseases (Butt *et al*., 2004).

The recent development of innovative methods for identifying microorganisms and pathogens has allowed for the detection of new pathogens in the environment (Scott *et al.,* 2002; Kong *et al*., 2002; Pommepuy and Le Guyader, 1999). Molecular techniques already used in clinical medicine are adapted for environment testing, assessing risks due to the dispersion of human pathogens in the sea. Thus, PCR, multiplex PCR, gene probes, and DNA fingerprinting techniques are now available to detect human enteric pathogens in seafood and seawater (Rose and Grimes, 2001). Contrary to traditional detection and enumeration methods that were time-consuming, mono-specific and ineffective in generating adequate information regarding public health (Wu, 1999), these techniques are rapid, sensitive, specific and cost-effective.

SOURCES OF CONTAMINATION

The presence of pathogens in the environment is predominantly a function of coastal urban and animal population densities and seasonal outbreaks or rainfall; it contributes to modifying the load of discharged pathogens. Rivers are the major routes, from land to sea, for the natural products of weathering and many man-made materials. They serve as a general conduit for materials from the land mass to the ocean. Three different types of fecal fluxes exist and are presented in Figure 1.

Permanent fecal fluxes from intestinal flora (estimated with *E. coli* and *Enterococcus)*, epidemic pathogenic fluxes resulting from human or animal outbreaks (ex: viruses) and endemic fluxes (ex: bacterial animal carrying) are likely to reach estuaries and coastal waters. Among others sources of contamination are boats or vessels, which could have a significant impact on water and sediment contamination (Seyfield *et al.,* 1997). Sobsey *et al*. (2003) reported that the fecal coliform concentration in marina water increased with travel freqency and boat occupancy during

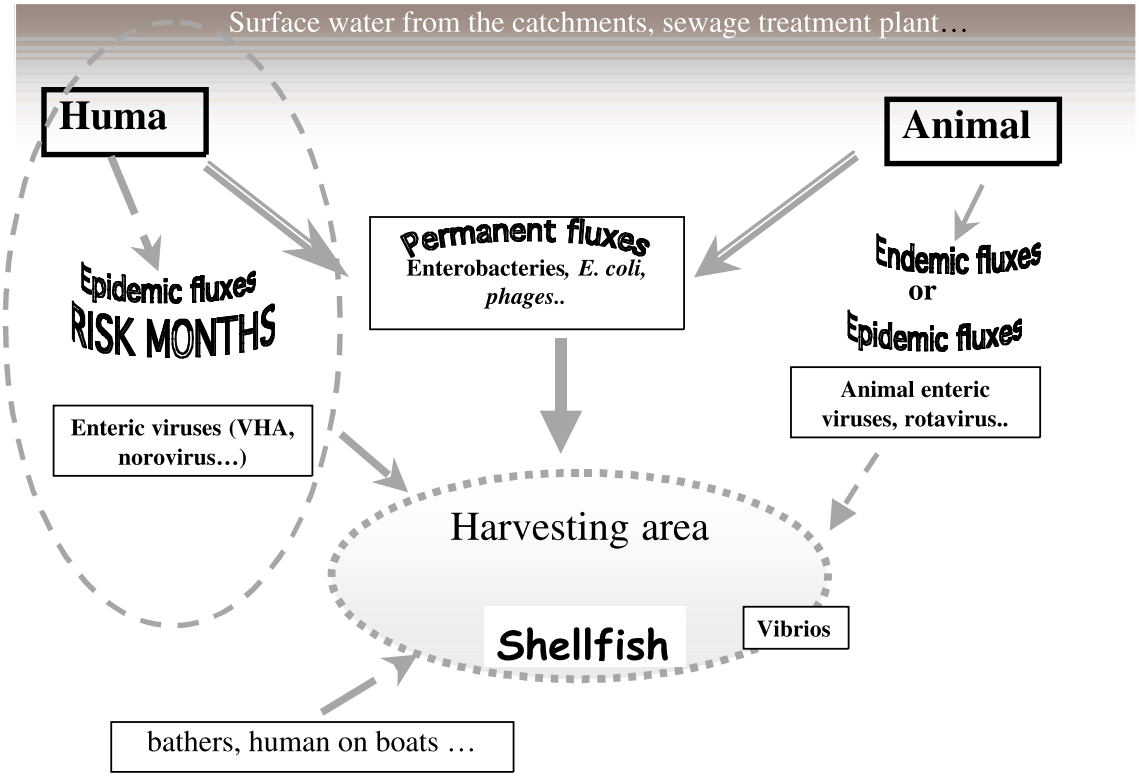

Fig. 1. Major origins of the main enteric pathogens detected in water.

the holiday weekends. Moreover, Dowel *et al*. (1995) clearly demonstrated that overboard disposal of sewage, currently practiced on harvesting areas, could be at the origin of fecal oyster contamination. To conclude on the possible origin of contamination, Gerba (2000) investigated the impact of bathers on pathogen concentrations in water. This author estimated that the fecal load sheds of bathers on weekends ranged from 10^{11} to 10^{16} viruses for 7185 bathers (normal and worst case conditions, respectively).

EMERGING PATHOGENS

Outbreak of gastroenteritis, *i.e*. pathogen excretion, is one of the major sources of viruses and bacteria in the environment (Payment and Hunter, 2001). Unfortunately, few data exist on the number of cases occurring in the different countries (Table 1). This number is highly variable and depends on the season and latitudes, health status of population, epidemic strains, etc. One estimates that approximately a single resident of developed countries is expected to become ill from an enteric infection at least once in the next 18 to 24 months, while residents of developing nations may experience between five and ten episodes per year. But the number of cases is generally largely underestimated, especially in developing countries. For example, in developed nations, over 98% of gastroenteritis are never reported to a physician (Lopman *et al*., 2003). Table 1 reports the published incidence rates of notified diseases observed in developed countries. The importance of viral outbreaks in comparison to other pathogen outbreaks has to be highlighted. Data from US indicate that, during seasonal epidemics, the main pathogen fluxes are those from viruses and are higher than those from bacteria (*E. coli* O157 - 20 000 cases/year reported in US population- or *Listeria* - 100 cases/year/ US population-) (Anonymous, 2000, 2001).

Human enteric viruses

In developed countries, outbreaks in the human population generally follow a seasonal pattern (Figure 2). The data from the French Network Survey (Reseau Sentinelle: wwwb3e.jussieu.fr) indicate that a peak of acute diarrhea is observed every winter from November to January. The

Table 1. Incidence rate of notified diseases in developed countries (100 000 of human population).

Notified diseases	Incidence rate /100 000 pop/year	References
Viral gastroenteritis	28 000.0	DeWit, 2001
Gastroenteritis (norovirus)	14 000.0	Lopman, 2003
Campylobacteriosis	100.4	"Foodborne", 1997
Salmonellosis	31.8	"Foodborne", 1997
Hepatitis A	11.7	"Foodborne", 1997
Shigellosis	5.6	"Foodborne", 1997
Vibriosis	2.5*	"Risk assessment", 2002
Yersiniosis	2.2	"Foodborne", 1997
Typhoid fever	0.5	"Foodborne", 1997
Listeriosis	0.4	"Risk assessment", 2002

* estimation from US data.

same results showing a regular pattern of seasonal outbreaks were also reported in other European countries and in the US (Lopman *et al*., 2003). Epidemiological and clinical investigations suggest a viral etiology for these winter epidemics. Norovirus represents more than 30% of the implicated viruses (Chikhi-Brachet *et al*., 2002).

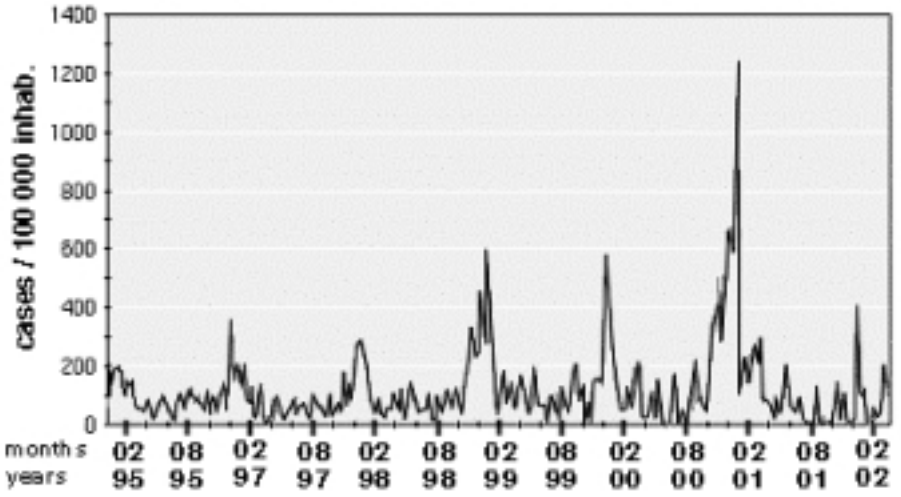

Fig.2. Occurrence of winter epidemics in France from 1996 to 2002.
(Sentiweb, www.b3jussieu.fr)

After multiplying in the gastrointestinal tract of man or animal, enteric viruses excreted in the feces are introduced into the environment (Gerba, 2000): viral concentration in stool could vary from 10^4 to 10^{10}/g for norovirus (Kageyama *et al*., 2003), 10^{10}/g for rotavirus (Griffin *et al*., 2003), 10^5 to 10^6/g for enterovirus (Hovi *et al*., 2001; Gerba, 2000), and 10^8/g for Hepatitis A virus (Yates and Yates, 1988). Asymptomatic excretion or infected persons shed persists for prolonged periods (2 -3 months) (Gerba, 2000). With a winter attack rate of 3% in the population and a norovirus concentration in stool of 10^6/g and 3 stools/day/patient, the flux for a population of 60,000 inhabitants could be estimated at $3x10^7$ virus per minute. (Pommepuy *et al*., 2004). Kohn *et al*. (1995) estimated that one person, with a viral diarrhea, may produce 10^9 viral particle/L of feces. This would be sufficient to contaminate $2x10^8$ L of seawater, *i.e*. the equivalent of the volume of an oyster harvesting area recently found implicated in outbreaks.

Griffin *et al*. (2003) estimated a viral concentration in wastewater (measured by cell culture), ranging from $1.82x10^1$ to 9.2 10^3/100ml in untreated sewage, and of less than 10/100ml in treated

sewage. Using molecular detection, the viral concentration in sewage was found to be higher than the one detected by cell culture assay: astrovirus concentration was ranging from 2.6 $x0^2$ to $3.6x10^4$ RT-PCR copies/100ml (Le Cann *et al.,* 2003) and adenovirus concentrations was 8.8 10^2 to 7.5 10^3/100ml in a treated domestic effluent (Jiang *et al*., 2001). Enterovirus concentration was estimated at $3.8x10^7$ RNA copies/100ml in raw sewage, 5.4 10^7 in sewage outfall, $2.3x10^7$ in sludge and $3.7x10^4$-$7x10^6$ in river (Schvoevre *et al*., 2001).

Pathogenic *Vibrio*

Among bacteria, the *Vibrio* family plays an important role in infections of waterborne or seafood diseases, especially in countries surrounded by warm marine waters (Colwell, 1978). Toxigenic *V. cholerae* O1 and O139 are the causative agents of cholera in developing countries, whereas non-O1 and non-O139 strains may also be pathogenic but rarely associated with epidemics (Anonymous, 2002). *Vibrio parahaemolyticus* has been recognized as a major cause of food-borne gastroenteritis in Japan and Eastern countries and linked to seafood consumption. In the United States, more than 700 cases of illness due to *V. parahaemolyticus* and associated to raw oyster consumption were reported between 1997 and 1998. However, only a few outbreaks have been reported in Australia (1990-1992) and in France (2001). The difference between the countries could be attributed to the ecology of this bacteria, which requires elevated temperatures to grow (>18°C). *V. vulnificus* associated with primary septicemia has also been detected in a variety of raw seafood products in Korea and Japan (Anonymous, 2002).

Endemic animal input, example of STEC, *Listeria spp., Campylobacter*

According to the socio-economic development, the bearing by healthy population can also be at the origin of weak and continuous contributions to the sea. Some rare information exists on the role of animal or human as pathogen carriers. Animals carry a large population of microbes in their intestinal tract, and contamination of the environment (water, soil, dust…), as well as of foods and food products, is widely reported (FDWP, 1997; Duffy *et al.,* 2001). Many microorganisms causing disease in humans are present in infected livestock: *Salmonella, Campylobacter jejuni, Listeria monocytogenes,* enterotoxigenic *E. coli,* rotavirus, etc. They occasionally produce symptoms of animal disease, but frequently colonize the intestinal tract. In intensive livestock catchments, the presence of pathogens in the animal could be at the origin of environmental contamination.

Escherichia coli commonly inhabits the intestine of man and animals, among which several pathogenic strains are at the origin of human diseases. Currently, more than 150 different serotypes are known to be associated with the production of verotoxins. The clinical features of STEC infection include diarrhea, which is often bloody, and may progress to severe haemolitic ureamic syndrome. The incidence of animal infections in Europe varies from 0.7/100 000 to 5.7/100 000 (Duffy *et al.,* 2001), according to geography. Healthy cattle typically carry STEC as a commensal in the gastrointestinal tract. Monthly excretion rates from cattle vary and summer peaks are observed. Same authors reported prevalence of carriage (presence/excretion) of STEC in absence of clinical symptoms. This bacteria is also able to colonize cattle for a long time (days/weeks). VTEC is present in environment samples in slaughterhouses, clarifiers of lagoons, and waste water treatment plants (Bouvet *et al*., 2002). In a review, Hancock *et al*. (2001), underline the role of water as the environment niche, because this bacteria has been found to persist and remain infective for at least 6 months in water through sediments. They can survive and even replicate during the warm season, for extended periods of– 2 to 8 weeks on grassland and pasture (Ogden *et al.,* 2001; Fukushima, 1999).

Listeria monocytogenes infects men as well as animals, and particularly the ovine races. It prevails in a sporadic or endemic way according to the techniques of breeding (Farber and Peterkin, 1991). The contamination could be contracted while ingesting plants; thus, the herbivores are mainly affected. The bacterial multiplication in silos has been demonstrated, and high concentrations were observed (10^2-10^6 UFC) when anaerobic conditions and proper pH were not monitored during storage (Al-Gazali and Al-Azawi, 1986; Stahl *et al*., 1996). For bovines, sheep and carnivores, enteritis can occur before any other clinical signs. Healthy carriers are frequently observed and can affect 20% of the cattle, depending on the nature of feeding and

on the seasons. High concentrations can be found in spreading and effluents: 10^5 -10^7 CFU/100ml. *Listeria monocytogenes* is able to colonize many environments because of its growth potential in a very wide range of temperatures (5°C to 42°C), with moderate nutritional requirements. Thus, commonly found in the grounds, water and plants, this bacteria proliferates in wet environments where it can persist for a long time under adverse conditions. Thus, lakes and rivers can be contaminated (Fenlon, 1999).

Thermophylic *Campylobacter*, and particularly *C. jejuni* and *C. coli*, are considered a leading cause of zoonoze enteric illness (Anonymous, 2002). Asymptomatic infection with *C. jejuni* and *C. coli* are frequent in adults. The clinical manifestation differs according to regions and strains, and includes fever, abdominal cramps and bloody diarrhea. These bacteria may be transferred to humans by direct contact with contaminated animals or ingestion of contaminated food or water. The principal reservoir of these bacteria is the intestinal tract of wild animals, especially wild birds and poultry (Naudeau *et al.*, 2001). However, it has also been isolated from domestic animals such as cattle, sheep, goats, etc. (Anon, 1999; 2003). Seasonality seems to influence the *Campylobacter* prevalence in retail chicken products in some countries where higher recovery rates were observed in contaminated foods during the warmer months. They grow at 37°C, but not below 32°C. Thus, as opposed to *Listeria*, *Campylobacter* could not survive for a long time in the humid soil or multiply in slaughter. However, this bacteria is able to evolve in a non culturable but viable form and survive for a long time in fresh water (Federighi *et al.*, 1998). These VNC were shown to be able to colonize animal and then recover their culturability (Talibart *et al.*, 1999; Cappelier *et al.*, 1999).

MICROORGANISMS SURVIVAL IN SEAWATER AND SEDIMENTS

The behavior of enteric microorganisms in the coastal environment depends on many factors which have been investigated for a long time. Recent reviews have been published (Rozen and Belkin, 2001; Troussellier *et al.*, 2000). The main factors affecting the behavior of microorganisms are, in addition to the organism itself and its physiological state, the physical and chemical characteristics of the marine environment (temperature, salinity, organic matter content, oxygenation, pH, etc.) and the atmospheric conditions (mainly sunlight radiation).

Most of the behavior investigations were done on *E. coli* and fecal coliforms, but a few authors also investigated *in situ* the behavior of pathogens discharged in marine environment. As an example, some T90s, mainly obtained from *in situ* experiments, are presented on Table 2. Different survival times were found according to the strains, recovery methods and field conditions (season, irradiance, temperature, depth, etc.) that were used. The persistence of viruses in marine water or other environments (fresh water, soil, crops), compared to that of *E. coli*, has to be highlighted (Carr, 2002).

Table 2. Examples of literature data on T90s in estuarine and marine waters. (Decimal reduction time is expressed in hours: minimum-maximum) 1. Montfort *et al.,* 2000; 2. Salomon and Pommepuy, 1991; 3. Troussellier *et al.,* 1998; 4. Callahan *et al.,* 1995; 5. Bosch, 1995; 6. Johnson *et al.,* 1996; 7. Arnal *et al.,* 2001; 8. Wait and Sobsey, 2001.

	Seawater 18-22°C	Seawater 4-5°C	Estuarine water 18-22°C	Estuarine water 4-5°C	References
Listeria innocua	5-45	54-89	6-24	57-96	1
Listeria monocytogenes	22-39	/	80	/	1
Escherichia coli	5-35	67-81	96-500	120-235	2, 3, 8
Salmonella tyohi	33-84	33-79	/	/	8
Salmonella panama	13-72	108-316	15-34	96-144	1
Poliovirus-1	10-72	158-170	/	/	4, 6, 8
F+RNA	60-76	/	/	/	4
Hepatitis A virus	72-672	/	/	/	4, 5, 7
Astrovirus	384-432	648-720	/	/	5

For a long time, authors had pointed out that sedimentation was involved in the decline of coliforms in the surface waters of the wastewater plume. Enteric microorganisms are mainly

accumulated in the surface layers of sediments, where concentration is more than 100 times higher than that found in supernatant water (Pommepuy *et al*., 1992). High concentrations occurred in the surface layer in comparison to the underlying sediment (Le Guyader *et al*., 1990). *Salmonella, E. coli, V. vulnificus, V. parahemolyticus,* enteric virus and other fecal microorganisms were detected in sediments (Hoi *et al*., 1998; Hielm *et al*., 1998).

Survival times in sediment were found to be very long and vary from several days (*E. coli*) to several weeks (fecal *streptococci*, *Salmonella*) or several months (viruses, *Clostridium*) (Le Guyader *et al*., 1990; Rhodes and Kator, 1988). Viruses adsorbed to sediment material remain infectious and survive for several months due to the protected effect of marine sediments in estuarine waters (Crenn *et al*., 1999).

DISCUSSION AND CONCLUSION

The release of fecal microorganisms and pathogens into the marine environment through sewage outfall and polluted rivers is of concern from the recreational standpoint (and a threat to important shellfish-growing areas). During the last decade, the risk assessment for bathing areas has been investigated (Haas *et al*., 1999; Fleischer *et al*., 1996; Ashboth *et al*., 1997; Kay *et al*., 1994). It was mostly estimated from epidemiological data and indicator concentrations in surface waters. More recently, Lopez-Pila and Szewyk (2000) established the minimum risk to which bathers are exposed, based on a dose-response relationship for rotavirus and the ratio of fecal coliform/rotavirus in water. Because the probability for infection by a single infectious unit is relatively high, these authors considered the rotavirus model to be very sensitive for assessing illness load. Assuming that a bather ingested 100ml water, the risk was estimated to be of 1.6/1000, if the mean value of lognormal distribution was 100 *E.coli*/100ml (95% percentile value: 6300 *E. coli*/100ml). From the same data, Haas (2002) estimated that the risk was two log lower. The risk assessment was also applied to viral contamination of shellfish (Lee and Jounger, 2002). An average virus exposure of 6 PFU per 60g of shellfish would lead to a risk estimated to be 31/1000 (Rose and Sobsey, 1993). For Bosch *et al*. (1994), the rotavirus risk varied from 15/1000 to 540/1000, depending if the shellfish was depurated or not.

To evaluate the risk due to the presence of these pathogens in the environment also depend on the infectious dose (Table 3).

Table 3. Microbial reported infection doses.

Microorganism	Estimated minimum infectious dose	References
Salmonella spp.	10^4- 10^{10}	Forsythe, 2002
Vibrio parahaemolyticus	10^6- 10^9	Forsythe, 2002
Campylobacter jejuni	10^2- 10^9 *	Black *et al.*, 1988
Listeria monocytogenes	10^2- 10^6	Anonym., 2000
Vibrio cholerae	10^3	Forsythe, 2002
Escherichia coli O157:H7	10-10^2	Forsythe, 2002
Hepatitis A	<10	Forsythe, 2002
Norovirus	< 10	Moe *et al.,* 2002

*Could be dose independent

The infectious dose varies with the strain, the age of patient or other parameters. Some pathogens are highly dangerous for man even at low concentrations (hepatitis A virus, *E. coli* O157:H7, *V. cholerae*), whereas others have to be ingested in high concentrations to be harmful (*V. parahaemolyticus*); others are highly infectious but not very dangerous (norovirus). For some pathogens, such as those in seafood, even a low contamination is unacceptable. Based on risk assessment models, maximum risks were estimated to be 1.3 infections per 100 swimmers (Colwell *et al*., 1996).

Viral zoonoses have become more evident in recent years. Unusual rotavirus strains in humans suggested animal transmission and co-infection of environmental samples by different strains

may enhance genome rearrangements and reassortiment (Palombo, 2002). The recent diagnosis of HEV infections in non travelers in developed countries raised the question of the source of infection (Halbur *et al.,* 2001). Molecular studies demonstrating sequence similarities among animal (swine, pigs, etc.) and human strains, and the detection of HEV in sewage in different countries, suggest that humans may become infected by contact with effluents of animal origin (van der Poel *et al.,* 2001; Clemente-Casares *et al.,* 2003). The zoonose risk can be extrapolated to other microorganisms. *E. coli* O157:H7 is a model of an emerging pathogen. Intensive livestock agriculture and the selective pressure during survival in the environment has led to horizontal transmission and accumulation of virulent factors (Duffy *et al*., 2001).

Different strategies were proposed to manage the risk. The conventional approach is to determine a better indicator than FC or *E. coli* for standards purposes. Thus, bacteriophages, *Clostridium* and enterococci are currently proposed (Lees, 2000; Griffin *et al*., 2001). But the relationship between pathogen and any indicator is uncertain. Other approaches directly addressing the presence of targeted pathogens in the environment had to be proposed. The following considerations could be investigated:

- *Development of molecular tools*: new genetic techniques provide the best challenge to detect pathogens in the sea and seafood. Genetic databases must also be expanded from existing databases (GeneBank, etc.).

- *Risk assessment*: quantitative information on pathogen concentration in the environment allows for better evaluation of the sanitary risk in coastal areas. Promising advances in molecular detection point out the possibility of directly detecting presence of pathogens in the environment. Real Time PCR, gene probes or biosensors are already proposed to detect the pathogens in food (Scott *et al.,* 2002; Pommepuy and Le Guyader, 2000). These methods applied to environment samples and added to genetic information on strains, (genotyping, molecular characterization, etc.) would help to identify the danger from pathogens among emerging pathogens.

- *Reduction of fecal input in coastal areas*. Microbial contamination in coastal areas is not a fatality. Recent studies carried out on water quality demonstrated the possibility to determine the main sources and to list the critical points in the catchments. Hydrodynamical models applied to these contaminants, even if they need further development and validation by databases, would serve as screening tools and provide rational bases for the evaluation of treatment levels. Thus, they will contribute to decision making on limiting the contamination.

- *Implementation of warning* systems: in the near future, as emphasized by Rose and Grimes (2001), an alert system based on gene chip technology will allow for direct detection of pathogens, preventing consumption of contaminated water or seafood. Expert systems for monitoring wastewater treatment plant are already available (Punal *et al*., 2002). They could be associated with neural networks to predict the wastewater inflow from sewage treatment plant or agriculture activities. These models calculated the hydraulic load and wastewater inflow able to reach the river and the coast when rainfall events occurred (Crowther *et al.,* 2002). Neural network models are developed to send data from different probes implemented in the environment and give real-time monitoring on rainfall, STP failures, salinity decreases and other parameters. This information could be gathered and connected with an early alert system implemented in catchments basin, bathing or harvesting areas (Crowther *et al*., 2002; EPA, 1999). Furthermore, in developed countries, information is already available on outbreaks occurring in the population. Associated with forecast information, salinity, STP failure and other, a warning system could lead to real-time assessment of water quality in bathing or harvesting areas.

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Linking the ecology, epidemiology and pathogenicity of *Vibrio cholerae:* a molecular genetic approach

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The bacterium *Vibrio cholerae* is both the causative agent of the infectious disease cholera and a common planktonic heterotrophic bacterium in aquatic ecosystems all over the world (Islam *et al*., 1997; Cottingham *et al*., 2003). Like many other emerging diseases, cholera is currently more prevalent and geographically widespread than in past decades, primarily as a result of human activities that affect the ecology of *V. cholerae* and/or increase human risk of encountering pathogenic strains (Colwell, 1996; Faruque *et al*., 1998; Ruiz *et al*., 2000; Harvell *et al*., 2002).

Molecular genetic tools have long been used to study pathogenic organisms in relation to the human host. For pathogens such as *V. cholerae*, this wealth of genetic information has the potential to be used in combination with ecological approaches to study the bacterium in an ecological context.

Using molecular genetic tools to address ecological questions about water borne pathogens can help mechanistically link human activities that affect aquatic ecosystems to disease dynamics and the potential for this disease to become endemic in new regions (Faruque *et al*., 1998; Faruque and Nair, 2002; Cottingham *et al*., 2003). We present here two examples of our use of this approach: investigations into the specific genetic mechanisms regulating attachment of *V. cholerae* to plankton, and an experiment investigating whole genome responses of *V. cholerae* to differences in aquatic nutrient levels.

Molecular mechanisms regulating environmental attachment and pathogenicity

Only two of the >200 known serogroups of *V. cholerae*, O1 (divided into classical and El Tor biotypes) and O139, are known to cause epidemic cholera (Faruque *et al*., 1998; Reidl and Klose, 2002). Attachment of ingested *V. cholerae* to the small intestine is necessary for the initiation of the disease and is mediated by several attachment factors including the toxin-coregulated pilus (Herrington *et al*., 1988).

Attachment provides a link between the natural behavior of the bacteria and their potential to be pathogenic, both because of the role of attachment in pathogenicity and because of the importance of attachment to cholera ecology (Figure 1). Seasonal outbreaks of cholera in tropical regions tend to correlate with seasonal plankton blooms in ponds and rivers used for drinking water (Figure 2), and *V. cholerae* is often found attached to zooplankton and phytoplankton surfaces, a behavior believed to enhance survival, growth, and transmission to human hosts (Huq

et al., 1984; Islam *et al*., 1993; Islam *et al*., 1999; Cottingham *et al*., 2003; Figures 1 and 2). The relative ability of different epidemic strains of *V. cholerae* to attach in different types of aquatic conditions may play a role in the shifting dominance among epidemic strains, and in the development of novel epidemic strains (Chiavelli *et al*., 2001; Cottingham *et al*., 2003).

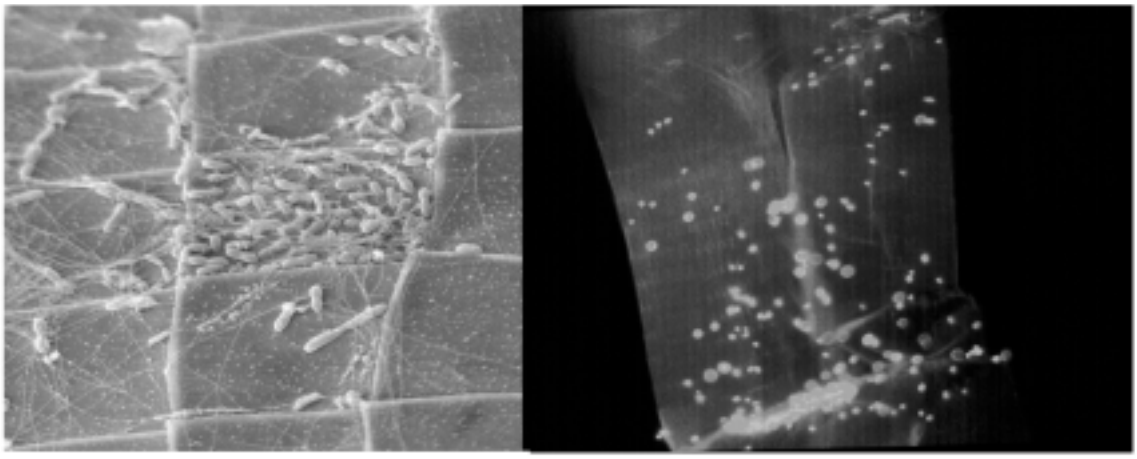

Fig. 1. Bacterial attachment to *Daphnia pulex* (a crustacean zooplankton). Left: SEM of *Vibrio cholerae* and other bacteria on a *D. pulex* carapace. Right: Fluorescent micrograph of *V. cholerae* on a *D. pulex* swimming appendage. 1. Major origins of the main enteric pathogens detected in water.

We are currently investigating specific genetic mechanisms that regulate *V. cholerae* attachment to plankton, all of which were initially studied for their potential role in intestinal colonization and resulting pathogenicity. Attachment assays with mutants indicate that multiple mechanisms regulate attachment of *V. cholerae* to plankton, that these mechanisms vary in strength and type among epidemiological strains and in the types of surfaces (hosts) with which they interact, and depend on environmental conditions such as nutrient level (Chiavelli *et al*., 2001; Taylor Lab, unpublished data). Several attachment mechanisms also mediate *V. cholerae* colonization of mammalian cells and two have been demonstrated to play a role in *V. cholerae* pathogenicity.

This approach to studying attachment using mutants can be expanded to address a number of questions relevant to bacterial ecology, epidemiology, land use, etc. For example, how are different attachment mechanisms regulated by environmental conditions such as nutrients, pH, and salinity? Do different attachment mechanisms contribute to differential survival, growth, prevalence of epidemiological vs. environmental strains? We have recently initiated studies comparing the persistence of wild type bacteria and attachment mutants in plankton communities to illuminate the role of attachment to plankton in *V. cholerae* dynamics.

MICROARRAY EXPERIMENT: EFFECTS OF NUTRIENTS ON WHOLE GENOME EXPRESSION OF *V. CHOLERAE*

Microarrays are a powerful new tool for studying an organism's response to its environment. A microarray consists of a glass microscope slide that contains up to several thousand cDNA or oligonucleotide spots that can be hybridized to differentially labeled products of reverse-transcribed RNA.

The availability of the complete genomic sequence for *V. cholerae* (Heidelberg *et al*., 2000) and a wealth of additional information resulting from extensive environmental, genetic and molecular studies facilitate our use of microarrays to look at *V. cholerae*. We can use microarrays to correlate environmental parameters with the molecular events associated with bacterial behavior and dynamics that may be relevant to its ecology, especially attachment, pathogenicity, and metabolism (Cottingham *et al*., 2003). The ability to evaluate simultaneous expression of the entire genome allows for a detailed, mechanistic interpretation of an organism's reaction to its environment and provides information that cannot be obtained from typical ecological techniques, such as those used to measure bacterial productivity, respiration, and enzyme production.

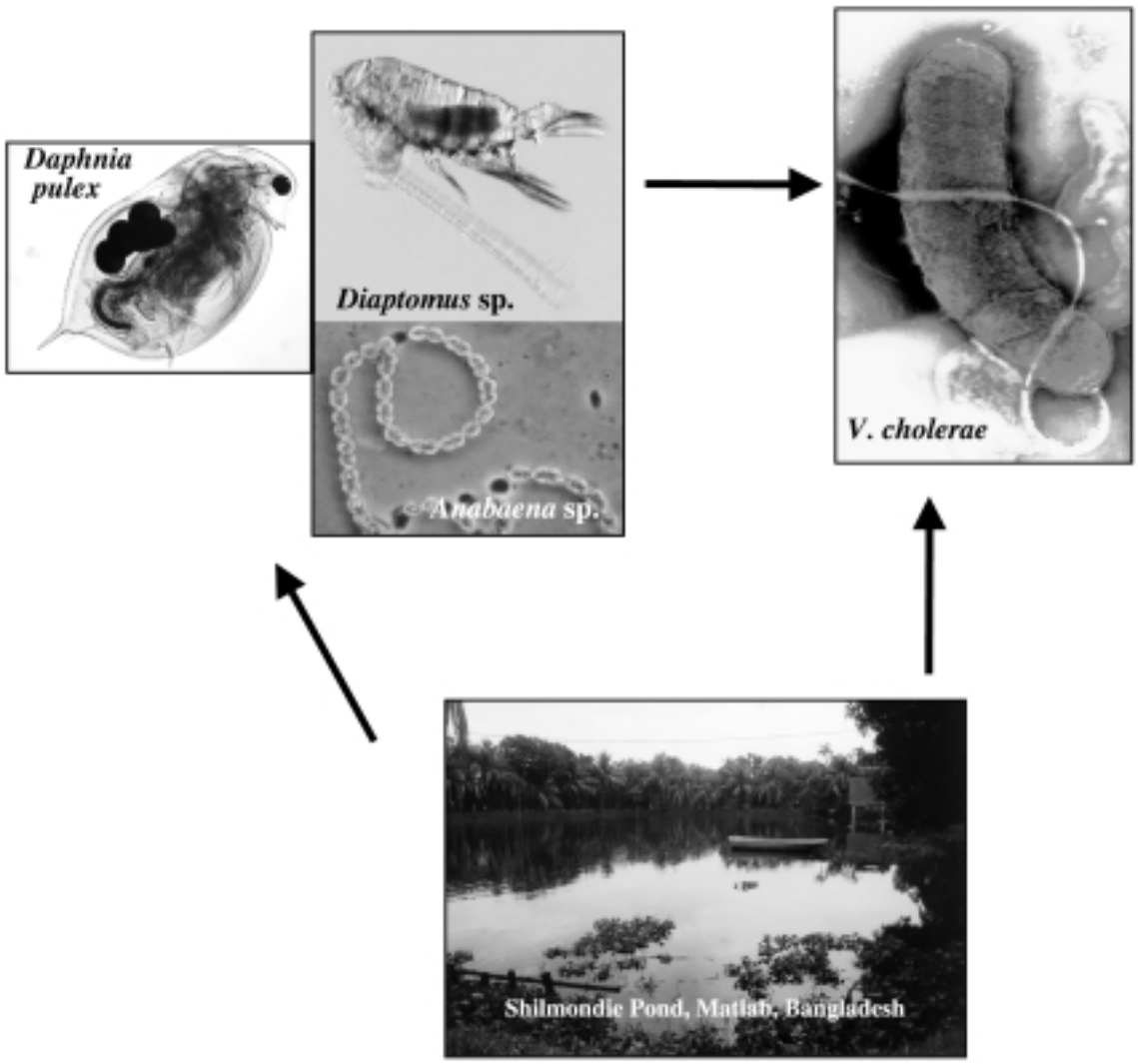

Fig. 2. Land use, climate, and seasonal effects on water conditions can affect *Vibrio cholerae* directly by affecting water quality, or indirectly by affecting zooplankton and phytoplankton communities that interact with the bacteria. Photograph credits: *D. pulex:* E. Kinney; *Anabaena:* K. Cottingham; *Diaptomus:* R. Thum; *V. cholerae:* T. Kirn, N. Bose, L. Howard; *Pond:* D. Chiavelli.

While aquatic conditions such as temperature, pH, nutrient availability, and plankton abundance all potentially play a role in regulating the abundance and pathogenicity of *V. cholerae*, the relative contributions and interactions of these factors have not been rigorously evaluated (Cottingham *et al*., 2003; Figure 2). We used microarrays to quantify the effect of varying levels of available carbon, nitrogen, and phosphorus on gene expression in an artificial freshwater medium for *V. cholerae* O1, El Tor, the globally predominant epidemic biotype.

Two major regulatory systems involved in pathogenesis showed increased gene expression in lower nutrient levels (Taylor lab, unpublished data). The first, the toxin-coregulated pilus (tcp) regulatory network, comprises four regulatory systems that control intestinal colonization and cholera toxin production (Attridge *et al*., 1996; Thelin and Taylor, 1996; Skorupski and Taylor, 1999). The second system, the *rfb* operon, generates the protective exopolysaccharide layer made by many enteric pathogenic bacteria that helps them survive passage through the stomach acid and survival in the intestine (Schnaitman and Klena, 1993).

The *tcp* regulatory network had 20 of 21 genes with increased expression in lower nutrient conditions, and for the *rfb* operon 18 of 19 genes had increased expression in lower nutrients. Interestingly, both surface colonization and production of a protective layer are physiological

responses that may allow bacteria to better survive harsh environmental conditions such as low nutrient levels. These results demonstrate a potential link between mechanisms mediating environmental survival and pathogenicity.

Other genes regulating factors known to mediate attachment to surfaces (attachment factors, flagellar genes, and chemotaxis genes) did not respond to nutrient levels. It is likely that for many of these factors a substrate cue may also be needed to induce attachment. Current and future microarray experiments will compare gene expression of *V. cholerae* attached to phytoplankton and zooplankton to gene expression of unattached *V. cholerae* both in the presence and absence of plankton.

CONCLUSION: MOLECULAR GENETIC TOOLS IN PATHOGEN ECOLOGY

The use of mutations can be a powerful aid to understanding how a specific phenotype of a pathogen contributes to its natural dynamics and to its ability to withstand environmental perturbations. Microarrays provide a way to monitor an organism's response to its environment at a molecular genetic level. As the genomes of more and more aquatic organisms are sequenced, and we continue to expand our knowledge of molecular genetics, these and other molecular genetic approaches can be invaluable to studying waterborne pathogens.

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