I - EXECUTIVE SUMMARY

This synthesis was written by all participants of the workshop under the coordination of Nicholas Fisher. Frédéric Briand, the Monograph Series Editor, reviewed and edited this chapter along with the entire volume.

ABSTRACT

CIESM convened in late September 2006 a workshop to review the state of knowledge relating contamination of the marine environment with public health. This opening chapter summarizes the participants' recommendations for future research so as to further consolidate our understanding of these complex issues and to improve mitigation strategies. Contaminants covered by the discussion included metals and metalloids, radioactive wastes, persistent organic compounds, new "emerging" organic compounds, and pathogenic microorganisms. Many of these contaminants enter coastal ecosystems through sewage treatment plants; some of them are known to affect not only the marine organisms that concentrate them but also human soncumers of seafood. Discussions also covered traditional and novel ways of monitoring, with particular attention to the Mediterranean Sea.

1. INTRODUCTION

There is ample evidence that toxic substances build up in marine food chains and are consumed in seafood, which is the principal pathway to humans for many of these contaminants. It is therefore critical to evaluate the bioconcentration of toxic chemicals in relation to human consumption and public health effects. These toxic substances include metals and metalloids, organic contaminants such as chlorinated hydrocarbons, polynuclear aromatic hydrocarbons, and pharmaceutical wastes, marine toxins largely associated with blooms of select algal species, and to a lesser extent radioactive wastes emanating from the nuclear fuel cycle. In addition, pathogenic microorganisms enter coastal waters via sewage disposal and many are known to have important public health consequences.

Metals and metalloids (e.g., mercury, lead, selenium, others) have been shown to reach concentrations in some harvested species that can result in impairment of nerve function in humans, cancer, and even death. Chlorinated hydrocarbons (e.g., DDT, PCBs, dioxins) are highly resistant to microbial and photochemical degradation and build up in marine food chains. Their consumption by humans can result in endocrine disruption, neurobehavioral, reproductive and developmental effects, and cancer. Polynuclear aromatic hydrocarbons can also build up in marine food chains and have carcinogenic effects in humans. Pharmaceutical wastes enter coastal waters in large quantities but their persistence in the environment, and bioaccumulation in marine organisms, are still largely unknown. In addition, human consumption of seafood containing toxins produced by harmful algal species can be subjected to severe neurobehavioral, respiratory, and gastrointestinal effects. Pathogens entering coastal waters largely through sewage treatment plant effluents and aquaculture operations can build up in marine bivalve molluscs as well as other shellfish and finfish, and can lead to gastrointestinal disorders, respiratory effects, various infections, and sometimes death.

Risks to human populations and to coastal ecosystems are increasing (1) with the advent of new technologies that result in enhanced discharges of increasingly complex (and poorly studied) chemicals into marine receiving waters; (2) as a result of increasing human population densities situated along coastlines worldwide; and (3) due to changing patterns of land use, including altered flow of rivers. Although global climate change is now well recognized, far less attention has been paid to the profound though gradual changes that are occurring in our oceans and coastal seas. Sea level rise, warming, altered ocean currents and ocean acidification will probably impact the bioavailability of contaminants, the occurrence and spread of infectious diseases and the biodiversity, structure and sustainability of marine ecosystems.

Given the increasing recognition of marine environmental contamination and their likely ties to public health, CIESM convened a workshop in Geneva, Switzerland, in September 2006 to discuss related issues, with special emphasis on the Mediterranean region where possible. Fifteen scientists from eight countries participated. The meeting was opened by Prof. Frédéric Briand, CIESM Director General, who presented the context of this brainstorming seminar, and gratefully acknowledged the local support of Prof. Daniel Ariztegui from the University of Geneva for his gracious hosting. He then invited Prof. Nicholas Fisher, Chair of CIESM Committee on Marine Biogeochemistry and workshop coordinator, to present the scientific objectives of the meeting.

2. TOXIC CHEMICALS AND THE RISK ASSESSMENT PROCESS

The influx, concentrations, speciation, and biological interactions of metals, radionuclides, and diverse organic contaminants in the Mediterranean and other marine ecosystems have been reviewed in recent CIESM workshops (CIESM, 2002c; 2004) and the reader is referred to those monographs for appropriate background information. Here we consider the potential toxic effects of these substances and how to evaluate risks to marine organisms and to human consumers of seafood.

One of the difficulties in forming balanced judgements of the impacts of marine pollution on human and other species is that human and ecological risk assessments are usually conducted separately. Although both human and ecological risk assessment protocols have a common goal, "to provide a quantitative estimate of the probability of an adverse effect occurring as a consequence of a contaminant release" (Newman, 1998), there are differences in regulatory practice, emphasis and technical feasibility that lead to differences in the endpoints commonly considered (Suter *et al*., 2004). Human health risk assessment is focused solely on the protection of human health and considers the effects on the individual, whereas ecological risk assessment also considers the consequences for populations, communities and ecosystems, giving less emphasis to the impact at the individual level.

There is an increasing recognition that risk assessments that fail to consider the interdependency of humans with their environment are unlikely to offer full protection to either human health or to nonhuman organisms, populations and ecosystems. Therefore, frameworks have been proposed to allow science-based approaches that combine the process of risk estimation for humans, biota and natural resources in one assessment (Suter *et al*., 2004). Guidelines for achieving this aim have been proposed by WHO in conjunction with OECD and the US EPA; they are listed in Table 1.

Table 1. WHO/IPCS guidelines for the integration of human and ecological risk assessment: priority research areas identified by Munns *et al.* (2003).

- Improved communication between health and ecological disciplines;
- Harmonization of exposure characterization and surveillance methods and models;
- Development of methods to facilitate comparison of risks among endpoints;
- Development of common endpoints across taxa;
- Improved understanding of mechanisms of effect at multiple scales of biological organization.

Current risk assessment models for carcinogens and many other potentially toxic chemicals are based on a default assumption of a linear, low-dose relationship such that even the smallest detectable levels represent a theoretical risk that is directly proportional to risks observed at higher doses experimentally or based on ecological or epidemiological studies. It is now possible to detect many contaminants at the part per trillion level or lower, more than six orders of magnitude lower concentrations than was possible only two decades ago. Yet it is still not possible to measure very small risks even in large populations, thus it is not possible to prove or disprove whether low doses carry a health risk or what the shape of the dose-response curve is in this very low dose region. However, recent studies of actual low-dose effects using new, highly sensitive techniques, such as gene expression changes, indicate that the dose-response for most chemicals is highly complex, multi-phasic, and clearly non-linear. Many of these studies also imply an absolute or practical threshold, below which there are no detectable biological effects, which is not reflected in the current default risk assessment models. It will be important, both from a health protective standpoint and from the pragmatic standpoint of resource allocation and prioritization that scientists and regulators begin to harmonize current scientific knowledge with the risk assessment process, since current models which are designed to be health protective can either over- or under-estimate risk of non-linear responses. The interaction of human activities with the Mediterranean marine environment represents an excellent model for incorporating scientific knowledge with science-based policy that is protective of human health, the environment, and important economic resources on the one hand, while balancing the wise and judicious use of resources based on knowledge and prioritization on the other.

Further complicating risk assessments is the observation that many contaminants elicit effects in which hormesis (or positive stimulation of biological events) is evident, particularly at low tissue concentrations of contaminants (see Pagano, this volume). These responses include increased growth rate, fertilization success, or enzyme activity. An unexplored, yet challenging subject in ecological and public health risk assessment may be ascribed to the impact of aquatic contaminants at subtoxic concentration ranges (Chapman, 2001). Given the non-linearity of biological responses to low concentrations of toxicants, observed for radiation and a wide variety of chemical stressors (Calabrese and Blain, 2005) to marine organisms (Stebbing, 1987; De Nicola *et al*., 2004; in press), risks assessments need to consider these phenomena before realistic appraisals can be made for public health following exposure to seafood with low contaminant levels. To better incorporate hormesis into guiding such risk assessments, further research is warranted in which bioassays consider a wide range of toxicant concentrations, including concentrations below the classical “no-effect-level” that may conceal the onset of hormetic effects. It becomes evident that careful consideration must be given to selecting appropriate controls, whose suboptimal quality should be compatible with the detection of either inhibitory or hormetic effect. Moreover, the assumption of “perfect” controls, i.e., lacking any adverse effect, may rarely exist in natural ecosystems or in humans.

2.1. Common end points and the application of biomarkers for evaluating toxicants

As we learn more about the molecular and cellular mechanisms that form the basis of biological processes, the sheer scale on which humans and other species, as diverse as fish, algae, and several invertebrates, share genes and cellular mechanisms in common becomes apparent. A further consequence of this is that they may be equally susceptible to the common mechanisms of action of toxic chemicals. Toxicological damage at the level of molecules (DNA, protein and lipid), cells (integrity of membranes, function of subcellular organelles) and tissues (necrotic or apoptotic lesions, structural changes) may be remarkably similar between quite diverse species. The limitations of biomonitoring are that it is impractical to keep measuring more and more chemical residues in the tissues of marine organisms. Often one chemical will influence the uptake of another and since exposure to pollutants usually occurs through complex mixtures, the interpretation of findings may be difficult, especially in relation to pollutant bioavailability. Perhaps the greatest limitation of biomonitoring is that it provides little insight into the adverse effects of pollutants on ecosystems and their components. There is a growing consensus that biomarker approaches offer greater potential in this regard. While consideration must always be given to exposure scenarios and to species differences in the patterns of uptake, metabolism and excretion, biomarkers (defined here as functional measures of exposure to stressors at the molecular, cellular or physiological level) can provide common, measurable endpoints to enable human and ecological risk assessments to be considered in a more integrated way.

Biomarkers are generally classified into four major categories: (a) internal dose, (b) biologically effective dose, (c) early biological effects and (d) susceptibility. This describes a continuum from exposure to disease, although different classifications may of course overlap. For example, blood or tissue contaminant concentrations, or metabolites, may be used to estimate exposure, altered enzyme activities may indicate biologically effective doses, susceptibility of pre-clinical effects, while pathological lesions provide evidence of adverse consequences (Galloway, in press).

Recent advances in molecular biology, diagnostic and analytical technologies mean that these techniques are increasingly becoming available for use with wildlife species, including mammals, fish and shellfish, with the promise of providing a more detailed insight into their toxicological and environmental responses to stressors. This has the potential to be very beneficial to the risk assessment process, particularly of marine contaminated areas, as it addresses the lack of mechanistic detail and genetic sequence data which have previously been available for the most popular invertebrate species used in aquatic ecotoxicology, such as the freshwater invertebrates *Chironomus riparius* or *Daphnia magna*, and the common marine mussel *Mytilus edulis*. Improved methods for determining exposure levels are also of particular relevance for assessing human exposure through contaminated food.

Key aspects of the biomarker approach that require attention are:

- that it is not sufficient to use a single biomarker as a surrogate for a specific pollutant. Experience has demonstrated that the use of suites of biomarkers to develop a weight of evidence approach is the most effective way to use biomarkers;
- biomarkers provide a means of assessing the integrated impact of pollutants and natural biotic and environmental stresses;
- biomarkers, used together with rapid chemical assessment methods, can provide an easy to use, rapid, inexpensive means of prioritizing among potential study sites. The utility of this approach has been demonstrated by the RAMP programs (Rapid Assessment of Marine Pollution) endorsed by UNEP, IOC and IMO. RAMP is a pilot program of the Global Oceans Observing System (GOOS);
- experience with the application of biomarkers has highlighted the need to validate methods with a broader range of species occupying different locations in food webs and reflecting a wide range of lifecycles and feeding mechanisms;
- the advent of genomic and proteomic approaches now presents the possibility of identifying changing patterns of gene and protein expression that may allow more effective identification of specific pollutant threats. Furthermore, such studies provide mechanistic insights that have relevance to the ontogeny of disease in humans;
- the global spread of infectious diseases, and the increasing recognition of the presence of bacteria, fungi and viruses in the marine environment clearly point to the value of biomarker approaches for assessing exposure to microbial threats and in the provision of early warning of the risk of the disease in biota and humans;
- many of the biomarkers used in environmental risk assessments are identical to those used in human risk assessment. This provides a potential means of linking environmental conditions with human health and well-being.

Further, there is a need in environmental studies to use multiple biomarkers in multiple species that are considered key components of marine ecosystems. To accomplish this, it will be important for scientists and other stakeholders to collectively choose model organisms that are good sentinel species, good keystone species, occupy key environmental niches that represent different trophic levels and different types of environments (estuarian, benthic, etc.) and which ideally are also amenable to genetic, physiological and other studies. Developing consensus on such model organisms would be a stepping stone to many different types of studies that could then link with each other to more effectively share information. Such common model species could be the basis of integrated monitoring programs, development and application of genomics tools and biomarkers, and development and application of these species as mechanistic models.

An example of a good model species currently under development is the killifish (*Fundulus heteroclitus*), which is a marine estuarian euryhaline teleost fish. It occupies a key position in the estuarian food web, and its health status at the individual and population level is highly representative of the condition of its food web and ecological niche. Thus it serves as both a keystone species and a sentinel species. Interestingly, its ability to rapidly adapt to changes in salinity is a result of complex physiological processes that are highly representative of several human physiological processes. Thus, it also serves as an excellent model for studying the human disease cystic fibrosis, for example, since both the human disease and killifish salt regulation are dependent on the same protein, CFTR. Genomics tools are now being developed (sequencing of the genome and expressed mRNA genome, development of DNA microarrays, proteomics analyses, etc.) for killifish that will make this an excellent model for environmental monitoring, physiological processes, and responses to multiple stressors. Careful selection of other key species using similar criteria would coalesce the scientific community around common models that could then be developed more rapidly and effectively than an ad hoc selection by individuals. Moreover, this would allow a single database to integrate many different layers of information from a wide variety of studies, as well as providing more robust spatial and temporal analyses.

2.2. Naturally occurring toxins

In addition to synthesized organic compounds, such as chlorinated hydrocarbon pesticides, PCBs, PAHs, and pharmaceutical wastes, potentially toxic metals that are greatly mobilized by man through mining and industrial activities, and radionuclides emanating from the nuclear fuel cycle and from weapons testing, there are other naturally occurring compounds that are highly toxic and that can affect both resident marine animals and human consumers of seafood. These include a broad array of toxins produced by marine organisms, but by far the compounds that have elicited the greatest public health consequences are those produced by harmful algal blooms (Landsberg, 2002). Compounds produced by a diverse microflora, but most particularly involving certain species of dinoflagellates and diatoms, can be accumulated by herbivores (e.g., shellfish) or fish and passed on to man. Massive fish kills have occasionally resulted, in addition to sick humans. Reported incidents of harmful algal blooms have increased markedly in recent years, and this is not simply due to increased vigilance of coastal waters (Smayda, 1990; Hallegraeff, 1993). The eutrophication caused by excessive nutrients introduced into coastal waters, frequently attributable to sewage treatment plant effluent as well as riverine input from increasingly fertilized watersheds, has greatly increased worldwide and has probably contributed to more frequent and prolonged harmful algal blooms (Anderson *et al.*, 2002). Some environmental factors have been identified that can trigger certain species to produce toxins, but there is still considerable uncertainty regarding this issue and no generalizations appear possible at this time. Reducing the magnitude of these harmful algal blooms and their toxicological consequences would appear to be tied to reducing nutrient discharge into coastal ecosystems, particularly in poorly flushed bodies of water.

2.3. Complex toxicant problems and possible resolution

A significant issue for all environmental and human health studies is the problem of multiple stressors. One example of this is the complex mixture of environmental contaminants that most ecosystems and most humans encounter. Most studies focus on a single stressor such as a chemical contaminant, pathogen or natural toxin. However, many recent studies using binary combinations of agents have demonstrated that they can interact additively, synergistically or antagonistically. We currently have little knowledge or predictive power to determine how two or more environmental agents may interact to lead to environmental or human health impacts. Yet there are examples where such interactions can lead to "tipping points" where significant effects occur that are poorly predicted by information of each agent in isolation. New studies should focus on those complex interactions that are either most likely to occur and/or have the greatest potential, based on current knowledge, to lead to non-additive interactions. Important mechanistic studies need to be conducted that will evaluate how, and to what extent, whole classes of chemicals are likely to interact with each other in order to develop a greater predictive capability for evaluating risks of organisms, including humans, to environmental contaminants. This will be even more important as emerging contaminants begin to build up in coastal waters, including new synthetic chemicals from industry, pharmaceuticals, etc., and emerging pathogens of concern.

There is a well-recognized continuous and increasing introduction of new contaminants into marine ecosystems, contaminants for which we often have no information on structure, persistence in the environment, reactivity for different environmental matrices, or biological effects (CIESM, 2004). While chemical analyses can help determine the exposure concentrations of xenobiotics, this type of analysis is limited because only known substances are analyzed, the interaction of mixtures of compounds is not taken into account, and currently the biological effects of these mixtures cannot be predicted. Moreover numerous organic contaminants can be metabolized and transformed into other compounds that can sometimes be more toxic than parent compounds (Schwarzenbach *et al*., 2003). The multi-contaminant (multi-sources, multi-classes) character combined with the temporal and spatial variability of their abundance, as well as complicating natural interactive forces (varying salinity, temperature, sunlight, etc.) makes more and more complex the evaluation of the contamination of environmental systems and the risk assessment procedure for the marine environment and for human health. In some cases, there may also be significant toxicological interactions between co-occurring toxins produced by blooms of some algae and these synthetic organic compounds or metals, but this is largely a matter of speculation at this time. Some progress may come with the development of screening methodology with no *a priori* selection of chemicals linking toxicity bioassays and chemical analyses. The new possibilities provided by the recent development in bioassay technology lead to the availability of a battery of specific bioassays able to determine the *in vitro* CMR (carcinogenic, mutagenic, reprotoxic) potency of chemical mixtures. By developing a “Toxicity Identification Evaluation” (TIE) type approach combining fractionation of total extracts, bioassays and chemical characterization of the toxic fraction, it may be possible to identify the toxic compounds present in the natural environment and in products related to human consumption.

Many of the new “emerging” contaminants are associated with discarded pharmaceutical chemicals, many of which enter coastal water through sewage treatment plants. Measured concentrations fluctuate between low ng l^{-1} to tens of μg l^{-1} levels, depending on the compound, location, and season. Most studies have assessed concentrations in the dissolved phase, but the particulate phase (phytoplankton, suspended abiotic particles) may also be important and may serve to introduce these substances into marine animals even more effectively than the aqueous phase (Fisher and Reinfelder, 1995). The bioaccumulation of most of these compounds in aquatic organisms, biological and abiotic breakdown products, and toxicity are just starting to be examined and need to be explored much more fully, as these could influence their impact on human health as well as the health of resident marine animals. Regarding antibiotics, bioaccumulation properties of some are known (e.g., in relation with their use in aquaculture) and there are EU norms fixing their maximum levels for products consumed by humans.

Genomics and proteomics represent two new experimental tools that can provide powerful information about the biological and toxicological responses of organisms, including humans, to environmental factors including chemicals of concern, pathogens, and other factors. Genomics provides the most sensitive tool for investigating very low level biological responses, and its ability to “fingerprint” unique responses to individual factors provides a means of both investigating a mechanism of action and using these tools for environmental monitoring and toxicant detection. Genomics can also be used to investigate complex and poorly understood phenomena such as non-monotonic dose responses including hormetic effects, potentially non-additive interactions of multiple stressors, and population shifts in response that are not obvious on the individual level. Proteomics provides a means of developing and validating powerful protein level biomarkers of exposure, susceptibility and effect that, in combination, can be used to assess complex environmental responses including changes in human response that presage subsequent health impacts. Collectively these tools rely on genomics information about the individual species under investigation. Thus, to most effectively apply genomics and proteomics to the marine environment it will be necessary to develop appropriate genomic information about model species under investigation.

3. Microbial pathogens in coastal marine environments

The contributions of Ingrid Brettar and Carlos A. Guzman to this section are gratefully acknowledged.

3.1. Pathogenic microorganisms of relevance to human health in the marine environment

Most human pathogens are found in coastal marine environments and not in open ocean habitats. This is commonly attributed to human activities on the coastline. Bacteria are the best studied of all known pathogens of marine origin (Belkin and Colwell, 2006). We know that only a very limited number of bacterial taxa contain pathogenic species (*Gammaproteobacteria*, *Epsilonproteobacteria*, *Firmicutes* and *Actinobacteria;* Brettar *et al*., this volume). Some of these bacteria are well known as indigenous marine bacteria, e.g., the *Vibrio* species, or freshwater bacteria from continental inputs, e.g., *Aeromonas* species (Monfort and Baleux, 1991), whereas others like *Salmonella* sp. and *Helicobacter pylori* are of allochthonous origin (Carbone *et al*., 2005). In addition to the well known pathogenic species or strains, "new species or strains" have to be considered as potential health threats when cases of infections start increasing (e.g., infections by *Mycobacterium marinum*, *Erysipelothrix rhusiopathiae*; Oliver, 2005) or when potentially pathogenic bacteria are detected in high numbers in marine coastal waters (e.g., *Arcobacter* spp.; Maugeri *et al*., 2004). Furthermore, the occurrence of pathogenic bacteria equipped with new virulence or antibiotic resistance genes through horizontal gene transfer (e.g., by bacteriophage transduction), or hybrid organisms generated from two genomes of non-pathogenic bacterial strains (Bisharat *et al*., 2005) can result in highly virulent bacteria representing a sudden and unexpected threat for human health. Thus, we have to take into account the threat by emergence and re-emergence on the species, subspecies, and clonal level, which is a major challenge for the provision of adequate detection tools and prevention measures.

The discovery of pathogenic viruses in marine environments is a relatively recent event with the first detection of *Coxsackievirus* in 1947 by Dalldorf and Sickles (for a review see Griffin *et al*., 2003). The four major families of pathogenic viruses found are *Adenoviridae*, *Caliciviridae*, *Picornaviridae* and *Reoviridae* (Brettar *et al*., this volume). These families comprise double stranded (ds) DNA, single stranded (ss) RNA and ds RNA viruses. All known pathogenic viruses that pose a significant health threat in the marine environment are transmitted via the fecal-oral route (hence their name as "enteric viruses") and are considered to be of allochthonous origin.

In addition to viruses, three genera of zoonotic protozoa, *Giardia*, *Cryptosporidium* and *Toxoplasma*, are detected in coastal waters (Fayer *et al*., 2004), sediments and shellfish. The origin of the protozoa is fecal matter of human or (mostly domestic) animals. Protozoa survive well as cysts that are small, buoyant, and resistant to most environmental influences, even to disinfectant, and remain infective in moist environments for long periods (up to a year); thus protozoan cysts are much more resistant to environmental stress than viruses and bacteria (Nasser *et al*., 2003). The occurrence of terrestrial protozoan species like *Cryptosporidium parvum* in shellfish indicates their land origin. Infection by these protozoa in the marine environment is observed for a large set of marine mammals.

Pathogens found in the marine environment are responsible for a broad spectrum of acute and chronic human diseases such as gastroenteritis, ocular and respiratory infections, hepatitis, myocarditis, meningitis, and neural paralysis. The origin of most marine pathogens is feces, with only a very few "autochthonous bacterial pathogens" (e.g., vibrios) able to grow in the marine coastal environment.

3.2. Ecology of marine pathogens

Survival of human pathogens in the marine environment is controlled by ecological mechanisms and environmental factors, such as grazing, solar radiation and temperature, organic matter availability, and suspended particle load. Infectivity might also depend on the physiological state and ecological fate of the pathogen and of the specific species that is infected. Concentrations of pathogens are strongly influenced by sewage discharge, ship traffic, and meteorological events like heavy rainfall. At present, despite a growing body of work in the field, there is still an inadequate understanding of the factors that affect the amount, survival, activity and infectivity of pathogens in marine systems.

Viruses and parasitic protozoa (in cyst form) are not considered capable of physiological responses to environmental stresses and can be regarded as almost inert biological particles in this respect. In contrast, bacteria are capable of activating different mechanisms to respond to such stresses and to survive in sub-optimal environmental conditions; a good example would be endospore formation by some gram-positive bacteria (e.g., *Clostridium*). In non-sporulating bacteria, cells arriving at the stationary phase of growth activate genetic cascades that enable an increased resistance to a number of stresses such as high or low temperature, increased salinity, osmotic shock and other environmental stressors (Jenkins *et al*., 1990; Ostling *et al*., 1993). Another adaptation mechanism more recently discovered to hostile environmental conditions is known as the Viable But Non Culturable (VBNC) state (Colwell and Huq, 1994; Lipp *et al*., 2002; Xu *et al*., 1982). A stressed bacterial cell that grows normally on culture media loses this capacity by entering the VBNC state, but can stay physiologically active and retain its virulence. The VBNC state was described for numerous allochthonous and autochthonous pathogenic bacteria. For autochtonous bacteria, such as *Vibrio*, the response would be an adaptation of the cell, for example in cold winter conditions. However, for an allochtonous pathogen such as *Salmonella*, the VBNC state can be considered an artifact of cellular degradation caused by environmental stresses that may lead to cellular lysis (Monfort and Baleux, 1994).

3.3. Ecological mechanisms supporting survival of pathogens in the marine environment

Organic nutrients improve the survival and growth conditions of bacteria in coastal waters. They can derive from algal growth or the land, introduced by river discharge, sewage, and runoff. The highest concentrations of organic nutrients are often found on the surface of organisms like algae or copepods. Attachment to these surfaces therefore provides a favorable environment for bacteria, including pathogenic bacteria like members of *Vibrio* genus (Lipp *et al*., 2002).

Attachment of microorganisms to particles in general increases survival and infectivity of bacteria and viruses (Brettar and Höfle, 1992; Griffin *et al*., 2003; Maugeri *et al*., 2004). For bacteria, improved nutrient conditions, shelter from grazing and UV irradiation improve the survival or growth conditions. For viruses, shelter from UV irradiation supports survival and infectivity. For protozoa, particle attachment seems to have little effect on survival in water. For all microorganisms, particles may serve as a vehicle for transfer to the sediment.

Attachment to plankton is of special relevance for the survival and growth of pathogenic bacteria in the marine environment. Attachment can occur to zooplankton and phytoplankton that both provide increased availability of organic nutrients and shelter from UV and grazing. For vibrios and other bacteria including fecal indicators, attachment to zooplankton increases survival, growth and distribution (via transport by the animal host and/or its feces) (Baffone *et al*., 2006; Maugeri *et al*., 2004; Signoretto *et al*., 2004).

Interactions with bivalves and other invertebrates provide protective and growth-promoting conditions for some pathogenic marine bacteria (e.g., vibrios) similar to attachment to plankton (Cavallo and Stabili, 2002). The resistance of some pathogenic bacteria and viruses to depuration procedures of edible bivalves is a further reason for the world-wide incidence of seafood borne diseases. Factors such as bacterial surface ligands, soluble hemolymph components, and the ability of some bacteria to activate distinct stress-signalling pathways involved in the hemocyte response are important in determining the bacterial fate within a bivalve host (Canesi *et al*., 2005; Pruzzo *et al*., 2005a). Interestingly, recent evidence shows that colonization ligands used by *V. cholerae* to interact with chitin-containing surfaces can be the same as those required for intestinal colonization (Kirn *et al*., 2005; Zampini *et al*., 2003). This observation points to a common link between the persistence of some potentially pathogenic bacteria in the environment and infection of the human host and suggests that virulence mechanisms of bacteria having environmental reservoirs may reflect adaptive mechanisms to the environment.

All pathogenic microorganisms show increased concentrations, prolonged survival and infectivity in sediments, especially surficial sediment (Fayer *et al*., 2004; Griffin *et al*., 2003; Brettar and Höfle, 1992). Thus, sediments that are resuspended by storms and ship traffic may introduce pathogens into the water column, particularly in lagoons and harbors. Reduced grazing and protection from UV irradiation could be important for the improved survival conditions of microorganisms, but further studies of the environmental factors that affect the survival and activity of pathogens in sediments are clearly needed.

3.4. Mechanisms reducing survival of pathogens in the marine environment

All pathogenic microorganisms are subject to grazing in the marine environment to some extent. Most relevant grazers are flagellates, ciliates, zooplankton such as cladocerans, filter-feeding bivalve molluscs, ascidians, polychaetes, and sponges. Grazing involves ingestion and digestion of the microorganisms. Bacteria are to some extent eliminated by grazing. Viruses are the least affected by grazing due to their small size.

Bivalves often exhibit a strong enrichment of pathogenic bacteria and viruses relative to the surrounding seawater (Miossec *et al*., 2000; Muniain-Mujika *et al*., 2003). Most gram-negative marine bacteria are considered to be well digested by bivalves (Birkbeck and McHenery, 1982), but many pathogenic bacteria, especially vibrios, are able to survive and even multiply within the bivalve gut (Charles *et al*., 1992; Pruzzo *et al*., 2005a).

UV radiation is able to destroy viruses and bacteria. Since UV radiation decreases with water depth and turbidity, bacteria and viruses in deeper water layers and/or particle-rich water are less stressed by UV radiation than in clear surface water. As well most pathogenic bacteria are more abundant at higher water temperature which promotes faster growth. With decreasing temperature, an increased fraction turns into a VBNC state (Huq *et al*., 2000) and the infectivity and survival rate may decrease. By contrast, viruses survive longer and display a higher rate of infectivity at lower temperature. Protozoa survive for a long time (as cysts up to a year or even longer) regardless of temperature (Fayer *et al*., 2004).

Bacteriophages can infect bacteria and lead ultimately to lysis and thus elimination of the bacterial cells. Bacteria occurring in the marine environment can either be infected before they enter the marine environment (e.g. in the intestine of a land animal, or in the sewage treatment facility) or be infected in the marine environment. Due to their specificity, the host spectrum of a specific phage is very restricted, usually to a specific bacterial species. Since the probability to be infected by a virus of the right specificity is dependent on the abundance/density of a bacterial host, bacteria with a high abundance in the coastal environment - such as vibrios or *Aeromonas* sp. - are much more likely to be infected and killed by viruses in the marine environment (Jiang *et al*., 2003). For bacteria unable to grow in the marine environment, infection by viruses of marine origin is unlikely; by contrast the presence of bacteriophages specific for enterobacteria can be used as a tracer for fecal contamination in shellfish or in coastal water (Dore *et al*., 2000).

3.5. Current hygienic problems in coastal environments

The main sources and transmission mechanisms of microbial contamination in coastal marine environments are sewage, run-off, floods, groundwater seepage, and river discharge. In specific areas, ship traffic, ballast water, and bathing represent additional sources. Well-treated sewage is intended to contain low numbers of bacteria, viruses and protozoa and the load of pathogenic microorganisms increases with decreasing treatment procedures/quality. Major problems arise during heavy rainfalls, storm events or floods that flush untreated or improperly treated sewage into rivers and coastal water, often leading to increased load of pathogens in coastal waters and increased incidence of disease (Miossec *et al*., 2000). It is noteworthy that treated and disinfected wastewater may still have a high load of pathogenic organisms, averaging 30 - 80% of that in untreated sewage (the lowest values for bacterial pathogens, the highest for protozoan cysts) (Harwood *et al*., 2005). Disposal of sewage sludge in agricultural areas, rivers and coastal regions can be another important source of pathogenic microorganisms.

Run-off carries pathogenic microorganisms from soils and urban surfaces and, depending on the strength of the rainfall, overloaded sewers, rain water collectors and sewage treatment facilities to rivers and coastal water. Depending on the season, this might be a threat to human health via contaminated seafood or seawater. Microbiologically contaminated groundwater may seep directly into coastal water or be transferred via river water. For viruses high transfer rates from groundwater aquifers, especially in limestone, to coastal water have been observed. Major contaminators of groundwater are households with septic tanks, livestock, and leaking sewage pipes. Rivers often carry high loads of pathogenic microorganisms, especially following rainfall events, to coastal waters.

In summary, feces of humans and domestic animals are major sources of pathogenic microorganisms entering coastal marine environments. The complete transport of feces to sewage treatment plants is difficult to achieve, especially during periods of heavy rain. Additionally, pathogenic bacteria, viruses and protozoa are somewhat reduced but not eliminated in sewage treatment plants (Harwood *et al*., 2005); those deposited in the sewage sludge are another source of contamination unless effective further treatment was achieved.

Furthermore, man-made eutrophication of coastal waters due to the addition of inorganic nutrients increases the production of algal biomass which in turn improves the survival of pathogenic bacteria by increasing the availability of organic nutrients and particles; the increasing particle load can also enhance the survival of pathogenic viruses. Global warming results in increased temperature of coastal waters, and this would also enhance survival of pathogenic bacteria but may diminish survival of viruses.

3.6. Emerging threats and potential mechanisms to increase virulence of microorganisms

Highly pathogenic microorganisms can either be directly introduced into the marine environment, or increase their pathogenicity in the marine environment. Major sources for introduction are sewage, with sewage derived from hospitals, and inadequately treated sewage especially from large urban areas representing a great threat. Additionally, ballast water has to be taken into account - not only for toxic algal blooms but also for the transfer of highly pathogenic microorganisms (Peperzak, 2005).

Mechanisms for increasing the pathogenicity for bacteria are i) horizontal gene transfer in aquatic breeding grounds, such as fish farms and overloaded sewage treatment plants, that allows acquisition of genes responsible for virulence or antibiotic resistance from other (usually related) bacteria or via bacteriophages, or - as recently shown - ii) formation of a hybrid highly pathogenic organism generated from two genomes of non-pathogenic bacterial strains (Bisharat *et al*., 2005). Horizontal gene transfer is more likely with increasing density of a specific pathogenic bacterial species or genus, e.g. more likely for vibrios than for enterobacteria and for sites of high concentration, such as gills of bivalves (Miller, 2001). Bacteriophages can serve as vehicles for nucleic acids of bacterial origin (genomic or plasmid DNA) that can lead to transduction of bacteria. A good example is the cholera toxin that is encoded by a lysogenic bacteriophage (Faruque *et al*., 1998). Chitinous surfaces are ubiquitous in marine environments on living crustaceans or their cast exoskeletons; it has been recently shown that *V. cholerae* interactions with such surfaces induce regulatory circuits that control transformation, thus providing bacteria another genetic mechanism to acquire new virulence or antibiotic resistance genes (Meibon *et al*., 2005).

The potential for contracting an infectious disease depends on different factors, including host susceptibility, the degree of exposure to the pathogens and the virulence of the agent. The increasing number of both aging and immunocompromised individuals, who are most susceptible to infections, emphasize the need for setting up control measures that are directed towards microorganisms with low virulence potential, such as *Aeromonas* spp.

It is now recognized that health issues, including those related to water borne diseases, are no longer simply a local problem but encompass global issues (Colwell, 2004). Natural events, which are now known to occur on a global scale, the worldwide moving of people (e.g., business, tourism) and goods, and human activities are largely responsible for the greatly enhanced global transport of microorganisms, including well known pathogens and novel pathogens such as SARS and extremely drug-resistant strains like *Mycobacterium tuberculosis* type XDR.

3.7. Possible solutions to improve the hygienic conditions of coastal seawater

Currently, hygienic quality and potential health risk from exposure to seawater and consumption of contaminated seafood are assessed by culturing indicator bacteria. This classical microbiological methodology relies on such methods as plate counts (CFU) of coliforms or plaque forming units (PFU). There are some serious deficiencies with these measurements there is no significant correlation between their outcome and the abundance of many microbial pathogens of non-fecal origin, nor do they provide valid identification of the pathogen. For this objective state-of-the-art molecular detection methodology has to be considered. Advanced methodology for environmental detection of microorganisms depends on: i) the type of microorganism, ii) the level of taxonomic resolution to be achieved, iii) the detection limit to be reached, and iv) the cost and time necessary to analyze samples. The approach now used widely is to analyze nucleic acids, extracted directly out of seawater, with a suite of molecular methods ranging from PCR and DNA array-based techniques to immuno-capturing and fluorescence *in situ* hybridization (FISH). Applying quantitative PCR techniques such as real-time PCR has enabled quantifying the pathogenic microorganisms in seawater without culturing them (Fey *et al*., 2004; González-Escalona *et al*., 2006). These advanced methods require considerable expertise and facilities and as yet are not routinely used for seawater analysis. There is clearly room for technological improvement, standardization, validation and automation of the molecular detection technology, particularly as it relates to pathogen detection in coastal waters. Validation of molecular detection methods for the environmental detection of microorganisms is also recommended for quality control of drinking water (OECD, 2003).

3.8. Interrupting the fecal-oral infection route by integrated coastal management

Transfer or storage of pathogens in sewage effluent seems to be a task that cannot be done without occasional high contamination of surface, ground water and coastal water. A solution to the risk of storage and transport would be a rapid on site extinction of these pathogens. Efficient procedures to reduce the load of pathogens (bacteria, viruses, protozoa, helminths) in human and animal waste in a short period of time (one to a few days) are those that include a step with heating of the waste, such as composting (>60°C, due to microbial heating) and the thermophilic production of biogas by methanogenesis (> 50°C). Critical to these procedures is that they reach high temperatures (above 50°C) for a sustained period (one to a few days). These hygienic measures for waste treatment are well established and could contribute to a healthier coastline; they are also profitable if the production of biogas is included in the treatment (Verstraete *et al*., 2002; Erickson *et al*., 2004).

A reduction of the transmission of zoonotic protozoa from pastures to river and coastal water can be most effectively achieved by “landscape solutions”, i.e. riparian buffer strips and reforestation, measures that reduce erosion and rapid run off, and improve the “filtering capacity” of the landscape. These measures will counteract in the same way non-point nutrient sources such as the transfer of nutrients (especially N and P) from the land to the ocean and thus reduce eutrophication of coastal water.

Generally, steps taken to reduce coastal eutrophication from point sources and non-point sources will likely reduce autochthonous pathogenic bacteria, such as vibrios, by removing a major source of organic nutrients for these bacteria (Mourino-Perez *et al*., 2003). Additionally, phytoplankton is the food for zooplankton - another major site for vibrio growth and dispersal. Thus, the hygienic treatment of waste and waste water, together with measures to reduce transfer of pathogens and nutrients from point and non-point sources, could provide the basis for an integrated coastal management scheme that could greatly reduce the occurrence and growth of pathogens in coastal environments and ensure safe bathing and seafood consumption.

3.9. Surveillance, epidemiology and integrated monitoring of marine-borne infectious diseases

Epidemiological studies of marine infectious diseases are lacking for almost all important aspects including the etiological agent, transmission routes, links between virulence properties and illness severity, number of cases and size of population exposed, etc. Therefore, a critical need and priority in the study and control of seawater-borne diseases is an epidemiologically structured network of surveillance based on contributions by public and private institutions, physicians and international health organizations.

Current monitoring of water quality in terms of pathogens is generally performed on a regular basis, mostly due to legal obligations. For example, at shellfish farming sites, monitoring of fecal indicators is usually done weekly. At bathing sites, this monitoring is often done weekly or bi-weekly during summer. However, the dynamics of pathogens in seawater, while still not well understood, suggests that marine bacterioplankton can rapidly respond to environmental changes within hours. Therefore, current sampling schemes are probably inadequate to predict human health threats originating from pathogens. An integrated monitoring approach appears to be the most promising solution to effectively protect human health considering all the parameters and forces influencing the presence, survival, activity and the actual infective dose of pathogens in seawater.

Integrated monitoring should combine all information available for a specific region, including GIS-based landscape data of the physical shape of the coastline and the underlying water body, physical, chemical and biological parameters (e.g., temperature, salinity, chlorophyll) integrated with dynamic hydrographical and meteorological data. To address public health issues, current and archived hygienic water quality data, such as indicator counts for *E. coli* and/or other indicator organisms, should be integrated and modeled in a dynamic way to understand the occurrence and spread of seawater-borne pathogens along the coast. More refined molecular data sets on the direct assessment of abundances of specific pathogenic viruses, bacteria and protozoa, should be added and, if their seasonal and spatial behavior has been assessed, can be modeled and integrated for the dynamic forecasting of health threats due to seafood consumption or bathing. Some particular sites (polluted or non polluted, closed or open bay) could then be used as test sites to validate dynamic monitoring models and their utility as early warning tool to prevent human infections. Other sites could be chosen for monitoring some pathogens in a continuous and long-term manner, to generate a sentinel network for the prevention of outbreaks of infectious diseases. These data should be integrated with environmental data specific to climate change so as to develop a long-term baseline for the assessment of health effects due to climate change.

An integrated monitoring approach could provide the basis for an early warning system capable of alerts regarding public health emergencies. Towards this end, remote sensing technologies with satellites could provide an important contribution with their dual capability of performing continuous environmental monitoring with coverage of large areas and acquiring/ transmitting data in real time. The establishment of possible correlations between specific parameters and the quantity and distribution of pathogens would be the basis for an early warning system on water-related health risks.

3.10. General conclusions and future research needs regarding pathogens

There is a rather limited knowledge on the microbiological principles governing the prevalence and pathogenesis of human microbial pathogens in the marine environment. The reasons for this lack of knowledge are that: i) the precise detection, identification and quantification of microorganisms in water are difficult and only possible with a combination of classical and molecular methods; ii) the virulence of waterborne pathogens varies with environmental conditions and the type of pathogen; and iii) the transmission of waterborne infections to humans via seawater is a complex process depending on the type of pathogen, infectious dose, immune status of the human and a multitude of other factors.

We foresee the following future research needs:

- development, validation and standardization of rapid detection methods for the most relevant pathogenic viruses and bacteria in seawater and seafood;
- abundance studies of major pathogens in the marine environment to elucidate their biogeography and their environmental control factors;
- thorough investigation of emerging marine pathogens including genomics, ecophysiology and host-pathogen-interaction;

- studies on the survival of pathogenic microorganisms in marine environments;
- studies on sanitation and sewage treatment conditions with respect to elimination / inactivation of pathogenic bacteria, viruses and protozoa;
- study of the epidemiology of seawater-borne and seafood-borne diseases originating from human pathogens of the marine environment.

4. CONTRIBUTION OF MARINE MONITORING PROGRAMS TO HUMAN HEALTH ISSUES

In order to protect the health of seafood consumers, many countries and international regulatory bodies have set guidelines for the maximum permissible levels of some well-known toxic contaminants (metals, chlorinated hydrocarbons, PAHs, etc.), bio-toxins and pathogens in seafood (e.g., European Commision, 2001). It is particularly important to monitor levels of key contaminants and pathogens in the coastal zone, where most fishery and farming activities are carried out and where contamination is most acute. Shellfish may harbor elevated contaminant and pathogen levels and serve as conduits for their passage to human consumers; they are therefore commonly monitored, as are coastal bathing waters during summer months for fecal indicator bacteria.

Monitoring contaminant levels in seafood is presently the only way to comply with seafood safety guidelines established by regulatory bodies. These measurements are also used to evaluate the extent to which remediation activities or pollutant reduction activities are effective. Monitoring can also reveal information for better understanding processes relating to the bioaccumulation and effects of contaminants in ecosystems (CIESM, 2002a,c). This has long been recognized, and research over the past two decades has attempted to combine field monitoring efforts with lab and field experimentation to provide explanations of field observations.

Biological indicators, or “sentinel species”, have been analyzed in monitoring program to determine baseline levels of key contaminants and assess their spatial and temporal trends in coastal regions. The choice of such indicator organisms is driven by two main constraints: relevance and feasibility. Probably the most widely applied bioindicator organism in the world is the mussel *Mytilus edulis*, or in the Mediterranean *Mytilus galloprovincialis*. This mussel has many attributes that make it suitable for assessing contaminant levels in coastal waters: it is sedentary, fairly ubiquitous, generally resistant to high levels of most commonly occurring contaminants (enabling it to inhabit heavily contaminated regions), and it accumulates contaminants from both the aqueous phase and from food such that tissue concentrations of contaminants reflect an integrated history of what the mussel experienced over extended periods of time. Perhaps most important, tissue concentrations of contaminants in the mussel clearly reflect the biologically available fraction of that contaminant. However, interpretation of mussel tissue data can be more complicated than that of plant material, since mussels eat food and can obtain some of their contaminants very appreciably from their diet (Wang and Fisher, 1997) in addition to the aqueous phase, which is the only source for such bioindicators as macroalgae. The advantages and limitations of using different biological indicators have been reviewed (Phillips, 1980). Regardless of which species are used, our understanding of the factors governing biological concentrations of contaminants often evolves over time, and so monitoring strategies must incorporate relevant experimental research to keep pace.

To ensure that meaningful spatial and temporal trends in contamination can be discerned using bioindicator organisms, every attempt should be made to use a single indicator species, since significant differences in contaminant bioaccumulation or degradation commonly exist between species. When the desired sentinel species is not naturally present in some regions, as is the case for the Mediterranean mussel (*Mytilus galloprovincialis*), it is possible to transplant bio-indicator organisms in caged devices, which is now a standard procedure (Andral *et al*., 2004). The transplantation/caging technique also helps minimize biological variability as it relies on the use of a calibrated batch of mussels from one single population deployed and then sampled at the same time in the different monitoring stations.

Passive samplers may hold promise to help understand the availability of dissolved, but not dietary, contaminants for marine organisms; they might therefore be useful for those contaminants for which diet is a relatively unimportant source term. Most use specific ligands and membranes with high affinities for certain classes of contaminants. These devices include the Semi-Permeable Membrane Device (SPMD) for hydrophobic compounds, the Polar Organic Compounds Integrative Sampler (POCIS system) for hydrophilic compounds, and Diffusion Gradient Thin Film (DGT) for metals. Once calibrated and validated, these tools could simplify logistics and sampling effort over time.

In addition to the problems associated with monitoring for xenobiotic organic compounds about whose environmental fate and bioaccumulation we still know relatively little, there are further complications involving the transformation of parent compounds to various degradation products, some of which are more toxic than the parent, such as some of the polycyclic aromatic hydrocarbons. Given the complications associated with monitoring for numerous contaminants, often co-occurring and often poorly understood, one approach that is increasingly adopted is to develop screening protocols that link chemical analyses with toxicity bioassays. The new possibilities provided by recent developments in bioassay technology lead to the availability of a battery of specific bioassays able to determine the *in vitro* CMR (carcinogenic, mutagenic, reprotoxic) potency of chemical mixtures. By developing a “Toxicity Identification Evaluation” (TIE) type approach combining fractionation of total extracts, bioassays and chemical characterization of the toxic fraction, it could be possible to identify the toxic compounds present in coastal regions and seafood.

As noted above, monitoring coastal regions should also consider discerning distributions of pathogenic microorganisms as well as chemical contaminants. In this context, different preservation procedures, such as deep freezing, can help archive samples in an appropriate format; given the uncertainties in this field at present and the current limitations of current methodologies, archiving samples for future analysis will likely prove to be of great importance. Archive samples accompanying current monitoring efforts would enable the establishment of an appropriate baseline for marine ecosystems, provide the possibility of tracking major contamination accidents or outbreaks of seawater-borne infections, and allow for future analyses of samples with new methods, new standardization or validation procedures. Ideally such an archiving program would be done on a large regional scale that would harmonize national efforts and establish common rules and procedures.

Satellite images are now broadly available to the scientific community: altimetry data, sea surface temperature and sea color (from which chlorophyll and suspended matter contents can be derived) are regularly provided with high resolution at the regional scale. Although satellite sensors can not measure dissolved contaminants or pathogens, these large-scale, high-frequency oceanographic data sets, coupled with circulation modeling, could guide monitoring strategies with regard to selecting sampling stations on appropriate spatial and temporal scales. Satellite surveys have already begun to evaluate water management strategies in France and are generally well suited for detecting plumes of visible contamination, such as oil spills and sewage treatment effluent, as well as chlorophyll levels in surface waters. The latter can provide evidence for the evolution of algal blooms following eutrophication, and all the attendant issues associated with these blooms. In time, it is expected that additional photosynthetic pigments can be detected, which may help identify blooms of potentially toxic phytoplankton (e.g., some dinoflagellates) that can impact the health and harvestability of some marine animals, including fish and shellfish. Satellites can also provide synoptic assessments of land use patterns in watersheds that may affect coastal ecosystems.

Such data should be stored in databases that are accessible by scientists and governmental agencies involved in monitoring efforts. These developments shall be considered in the European program GMES (Global Monitoring for Environment and Security). GMES is a joint initiative from the European Commission and the European Space Agency for the purpose of providing, on a sustained basis, reliable and timely services related to environmental and security issues in support of public policy makers’ needs. It will cover global, national and regional issues relating to climate, environment management and civil security. GMES aims to coordinate existing systems, produce services of guaranteed validity, and ensure their future continuity. It will stimulate capacity growth through the creation of new observation sensors and a wide variety of added-value services. GMES will be based on a comprehensive earth observing system, including different types of data (space, airborne, *in situ*), efficient data management and information sharing, and organized services deployable on a European scale.

The first operational services will deploy in 2008. GMES has been declared as the main European contribution to the GEOSS (Global Earth Observation System of Systems) whose implementation plan was signed in February 2005 in Brussels by 60 countries and 40 Organizations. Initial services were defined to establish the foundations of GMES and include the marine and coastal environment (including pollution, oil spills, water quality).

The workshop allowed a first attempt for scientists to identify specific needs for a reference data base on coastal areas (Table 2).

Table 2. Specific needs for satellite data for understanding coastal contamination.

Application	Spatial resolution	Time frequency	Accuracy	Need and priority
Sea Data				
Coastline	10 m	Once a year May be more for change detection	3 m	High
Bottom color	10 m	Once a year or on request	low	High Sediment accurate location Algae location Need of separation between mineral and vegetation
Classification of mineral/biological matter		Once a month or on request		High importance just to separate biological from mineral matter
Water color White phenomenon Green Red	5 -10 m	During summer		High for particular phenomenon
Chlorophyll measurements	10-30 m	Once a month or on request	mg/m^3	Only if there is a continuous spectrum from blue to NIR (1.2 µm) Useful obviously for microbiologist
Suspended matter	10-30 m	Once a month or on request	mg/m^3	High Particles distribution
Turbidity	10-30 m	Once a month or on request		Important regarding the illumination of the bottom by the sun
Location of sand bank	10-30 m			No
Trace Metals				OK for Mo, Mn, Fe
Coastal currents	50 m	Once a month or on request		Highly important
Local pollution (e.g., oil spill) Sewage Floods events Meteorological events River plumes	10-30 m	On request		Important
Water temperature	50 m	Once a month or on request	One degree resolution at least 0.1 be better	High
Aerosols concentration from sea level up to 500 m.	50 m			High
Land Data				
Land use	10-30m	Once a year		High Catchments area (basin versant) of the Mediterranean Sea
Location of ports, dikes, towns sewage, farms, pipelines, industries	5-10m	Once a year		High
3D Data				
Bathymetry	10-30m	Once a year	No need of a high accuracy	Low
DEM	10-30m	Once a year		High
Connection Bathymetry-DEM	10-30m			High
Hydrographic network				High to Low, depending on the activity
Temporal survey				
For pollution detection and survey	Every 6 hours Daily data Weekly data			
What geographical surface?	10km from the coast offshore			
What kind of phenomenon?	Pollution			

5. SPECIFIC MEDITERRANEAN ISSUES

The Mediterranean Sea represents a unique marine environment as it is a large semi-enclosed oligotrophic system with its own unique oceanographic properties, including a water temperature that remains ≥ 12ºC, a fact that will affect growth and survival of pathogens, degradation of organic compounds, etc. It is surrounded by countries with greatly varying degrees of industrialization and agricultural development. A key feature of the Mediterranean region is the uneven distribution of expertise in the fields of environment and human health. The emergence of new threats and new technologies to monitor and mitigate impacts highlight the need for building human capacity in these areas, especially in countries where economic challenges are greatest, notably along the southern Mediterranean coast. Areas in which training programs are urgently needed include environmental informatics, integrated environment and human health risk assessment, detection and monitoring of infectious diseases, and in the use of new tools. Training should involve exchange visits among riparian states. A positive outcome would be a transfer of knowledge and increased understanding and effective working relationships among scientists in the region.

There is no doubt that the amount of information available regarding the environment and human health in the Mediterranean region is increasing exponentially, although considerable gaps in our knowledge on a wide range of topics still remain. It is timely to consider synthesizing data around certain themes like contaminant distribution, exposure to infectious diseases, disease outbreaks, etc. It would then be possible to investigate the relationships among a wide range of phenomena and develop integrated strategies to protect environment and human health. There is a need for a new Mediterranean bioinformatics program consisting of databases integrating data from diverse studies in common formats that can be analyzed in a multi-variate manner. Such integration will also require new analytical tools including complex modeling and systems biology approaches that will be useful to other similar large system studies.

Social patterns may also influence the extent to which health-threatening contaminants enter the Mediterranean Sea and affect public health. There are striking increases in local populations during the summer tourist season, for example, during which time more contaminants enter coastal systems through sewage (treated and untreated) and more seafood is consumed. Because there are substantial cultural differences among the human populations surrounding the Mediterranean, different lifestyles and food habits must be carefully examined when addressing public health issues related to seafood consumption. For example, religious restrictions forbid some populations from eating shellfish and almost all harvested or cultivated production is exported to EU countries. Hence, while no direct health concerns may exist for local populations, seafood safety must comply with EU guidelines and regulations. Even desalinated Mediterranean water can serve as a source of drinking water (and associated contaminants) in some areas, and again variations among cultures are pronounced. It is therefore appropriate to use critical pathway analysis, first established for radioactive wastes, to determine the health risks related to specific types of chemical contaminants and pathogens for the most vulnerable groups in the Mediterranean.

I - EXECUTIVE SUMMARY

This synthesis was written by all participants of the workshop under the coordination of Nicholas Fisher. Frédéric Briand, the Monograph Series Editor, reviewed and edited this chapter along with the entire volume.

ABSTRACT

CIESM convened in late September 2006 a workshop to review the state of knowledge relating contamination of the marine environment with public health. This opening chapter summarizes the participants' recommendations for future research so as to further consolidate our understanding of these complex issues and to improve mitigation strategies. Contaminants covered by the discussion included metals and metalloids, radioactive wastes, persistent organic compounds, new "emerging" organic compounds, and pathogenic microorganisms. Many of these contaminants enter coastal ecosystems through sewage treatment plants; some of them are known to affect not only the marine organisms that concentrate them but also human soncumers of seafood. Discussions also covered traditional and novel ways of monitoring, with particular attention to the Mediterranean Sea.

1. INTRODUCTION

There is ample evidence that toxic substances build up in marine food chains and are consumed in seafood, which is the principal pathway to humans for many of these contaminants. It is therefore critical to evaluate the bioconcentration of toxic chemicals in relation to human consumption and public health effects. These toxic substances include metals and metalloids, organic contaminants such as chlorinated hydrocarbons, polynuclear aromatic hydrocarbons, and pharmaceutical wastes, marine toxins largely associated with blooms of select algal species, and to a lesser extent radioactive wastes emanating from the nuclear fuel cycle. In addition, pathogenic microorganisms enter coastal waters via sewage disposal and many are known to have important public health consequences.

Metals and metalloids (e.g., mercury, lead, selenium, others) have been shown to reach concentrations in some harvested species that can result in impairment of nerve function in humans, cancer, and even death. Chlorinated hydrocarbons (e.g., DDT, PCBs, dioxins) are highly resistant to microbial and photochemical degradation and build up in marine food chains. Their consumption by humans can result in endocrine disruption, neurobehavioral, reproductive and developmental effects, and cancer. Polynuclear aromatic hydrocarbons can also build up in marine food chains and have carcinogenic effects in humans. Pharmaceutical wastes enter coastal waters in large quantities but their persistence in the environment, and bioaccumulation in marine organisms, are still largely unknown. In addition, human consumption of seafood containing toxins produced by harmful algal species can be subjected to severe neurobehavioral, respiratory, and gastrointestinal effects. Pathogens entering coastal waters largely through sewage treatment plant effluents and aquaculture operations can build up in marine bivalve molluscs as well as other shellfish and finfish, and can lead to gastrointestinal disorders, respiratory effects, various infections, and sometimes death.

Risks to human populations and to coastal ecosystems are increasing (1) with the advent of new technologies that result in enhanced discharges of increasingly complex (and poorly studied) chemicals into marine receiving waters; (2) as a result of increasing human population densities situated along coastlines worldwide; and (3) due to changing patterns of land use, including altered flow of rivers. Although global climate change is now well recognized, far less attention has been paid to the profound though gradual changes that are occurring in our oceans and coastal seas. Sea level rise, warming, altered ocean currents and ocean acidification will probably impact the bioavailability of contaminants, the occurrence and spread of infectious diseases and the biodiversity, structure and sustainability of marine ecosystems.

Given the increasing recognition of marine environmental contamination and their likely ties to public health, CIESM convened a workshop in Geneva, Switzerland, in September 2006 to discuss related issues, with special emphasis on the Mediterranean region where possible. Fifteen scientists from eight countries participated. The meeting was opened by Prof. Frédéric Briand, CIESM Director General, who presented the context of this brainstorming seminar, and gratefully acknowledged the local support of Prof. Daniel Ariztegui from the University of Geneva for his gracious hosting. He then invited Prof. Nicholas Fisher, Chair of CIESM Committee on Marine Biogeochemistry and workshop coordinator, to present the scientific objectives of the meeting.

2. TOXIC CHEMICALS AND THE RISK ASSESSMENT PROCESS

The influx, concentrations, speciation, and biological interactions of metals, radionuclides, and diverse organic contaminants in the Mediterranean and other marine ecosystems have been reviewed in recent CIESM workshops (CIESM, 2002c; 2004) and the reader is referred to those monographs for appropriate background information. Here we consider the potential toxic effects of these substances and how to evaluate risks to marine organisms and to human consumers of seafood.

One of the difficulties in forming balanced judgements of the impacts of marine pollution on human and other species is that human and ecological risk assessments are usually conducted separately. Although both human and ecological risk assessment protocols have a common goal, "to provide a quantitative estimate of the probability of an adverse effect occurring as a consequence of a contaminant release" (Newman, 1998), there are differences in regulatory practice, emphasis and technical feasibility that lead to differences in the endpoints commonly considered (Suter *et al*., 2004). Human health risk assessment is focused solely on the protection of human health and considers the effects on the individual, whereas ecological risk assessment also considers the consequences for populations, communities and ecosystems, giving less emphasis to the impact at the individual level.

There is an increasing recognition that risk assessments that fail to consider the interdependency of humans with their environment are unlikely to offer full protection to either human health or to nonhuman organisms, populations and ecosystems. Therefore, frameworks have been proposed to allow science-based approaches that combine the process of risk estimation for humans, biota and natural resources in one assessment (Suter *et al*., 2004). Guidelines for achieving this aim have been proposed by WHO in conjunction with OECD and the US EPA; they are listed in Table 1.

Table 1. WHO/IPCS guidelines for the integration of human and ecological risk assessment: priority research areas identified by Munns *et al.* (2003).

- Improved communication between health and ecological disciplines;
- Harmonization of exposure characterization and surveillance methods and models;
- Development of methods to facilitate comparison of risks among endpoints;
- Development of common endpoints across taxa;
- Improved understanding of mechanisms of effect at multiple scales of biological organization.

Current risk assessment models for carcinogens and many other potentially toxic chemicals are based on a default assumption of a linear, low-dose relationship such that even the smallest detectable levels represent a theoretical risk that is directly proportional to risks observed at higher doses experimentally or based on ecological or epidemiological studies. It is now possible to detect many contaminants at the part per trillion level or lower, more than six orders of magnitude lower concentrations than was possible only two decades ago. Yet it is still not possible to measure very small risks even in large populations, thus it is not possible to prove or disprove whether low doses carry a health risk or what the shape of the dose-response curve is in this very low dose region. However, recent studies of actual low-dose effects using new, highly sensitive techniques, such as gene expression changes, indicate that the dose-response for most chemicals is highly complex, multi-phasic, and clearly non-linear. Many of these studies also imply an absolute or practical threshold, below which there are no detectable biological effects, which is not reflected in the current default risk assessment models. It will be important, both from a health protective standpoint and from the pragmatic standpoint of resource allocation and prioritization that scientists and regulators begin to harmonize current scientific knowledge with the risk assessment process, since current models which are designed to be health protective can either over- or under-estimate risk of non-linear responses. The interaction of human activities with the Mediterranean marine environment represents an excellent model for incorporating scientific knowledge with science-based policy that is protective of human health, the environment, and important economic resources on the one hand, while balancing the wise and judicious use of resources based on knowledge and prioritization on the other.

Further complicating risk assessments is the observation that many contaminants elicit effects in which hormesis (or positive stimulation of biological events) is evident, particularly at low tissue concentrations of contaminants (see Pagano, this volume). These responses include increased growth rate, fertilization success, or enzyme activity. An unexplored, yet challenging subject in ecological and public health risk assessment may be ascribed to the impact of aquatic contaminants at subtoxic concentration ranges (Chapman, 2001). Given the non-linearity of biological responses to low concentrations of toxicants, observed for radiation and a wide variety of chemical stressors (Calabrese and Blain, 2005) to marine organisms (Stebbing, 1987; De Nicola *et al*., 2004; in press), risks assessments need to consider these phenomena before realistic appraisals can be made for public health following exposure to seafood with low contaminant levels. To better incorporate hormesis into guiding such risk assessments, further research is warranted in which bioassays consider a wide range of toxicant concentrations, including concentrations below the classical "no-effect-level" that may conceal the onset of hormetic effects. It becomes evident that careful consideration must be given to selecting appropriate controls, whose suboptimal quality should be compatible with the detection of either inhibitory or hormetic effect. Moreover, the assumption of "perfect" controls, i.e., lacking any adverse effect, may rarely exist in natural ecosystems or in humans.

2.1. Common end points and the application of biomarkers for evaluating toxicants

As we learn more about the molecular and cellular mechanisms that form the basis of biological processes, the sheer scale on which humans and other species, as diverse as fish, algae, and several invertebrates, share genes and cellular mechanisms in common becomes apparent. A further consequence of this is that they may be equally susceptible to the common mechanisms of action of toxic chemicals. Toxicological damage at the level of molecules (DNA, protein and lipid), cells (integrity of membranes, function of subcellular organelles) and tissues (necrotic or apoptotic lesions, structural changes) may be remarkably similar between quite diverse species. The limitations of biomonitoring are that it is impractical to keep measuring more and more chemical residues in the tissues of marine organisms. Often one chemical will influence the uptake of another and since exposure to pollutants usually occurs through complex mixtures, the interpretation of findings may be difficult, especially in relation to pollutant bioavailability. Perhaps the greatest limitation of biomonitoring is that it provides little insight into the adverse effects of pollutants on ecosystems and their components. There is a growing consensus that biomarker approaches offer greater potential in this regard. While consideration must always be given to exposure scenarios and to species differences in the patterns of uptake, metabolism and excretion, biomarkers (defined here as functional measures of exposure to stressors at the molecular, cellular or physiological level) can provide common, measurable endpoints to enable human and ecological risk assessments to be considered in a more integrated way.

Biomarkers are generally classified into four major categories: (a) internal dose, (b) biologically effective dose, (c) early biological effects and (d) susceptibility. This describes a continuum from exposure to disease, although different classifications may of course overlap. For example, blood or tissue contaminant concentrations, or metabolites, may be used to estimate exposure, altered enzyme activities may indicate biologically effective doses, susceptibility of pre-clinical effects, while pathological lesions provide evidence of adverse consequences (Galloway, in press).

Recent advances in molecular biology, diagnostic and analytical technologies mean that these techniques are increasingly becoming available for use with wildlife species, including mammals, fish and shellfish, with the promise of providing a more detailed insight into their toxicological and environmental responses to stressors. This has the potential to be very beneficial to the risk assessment process, particularly of marine contaminated areas, as it addresses the lack of mechanistic detail and genetic sequence data which have previously been available for the most popular invertebrate species used in aquatic ecotoxicology, such as the freshwater invertebrates *Chironomus riparius* or *Daphnia magna*, and the common marine mussel *Mytilus edulis*. Improved methods for determining exposure levels are also of particular relevance for assessing human exposure through contaminated food.

Key aspects of the biomarker approach that require attention are:

- that it is not sufficient to use a single biomarker as a surrogate for a specific pollutant. Experience has demonstrated that the use of suites of biomarkers to develop a weight of evidence approach is the most effective way to use biomarkers;
- biomarkers provide a means of assessing the integrated impact of pollutants and natural biotic and environmental stresses;
- biomarkers, used together with rapid chemical assessment methods, can provide an easy to use, rapid, inexpensive means of prioritizing among potential study sites. The utility of this approach has been demonstrated by the RAMP programs (Rapid Assessment of Marine Pollution) endorsed by UNEP, IOC and IMO. RAMP is a pilot program of the Global Oceans Observing System (GOOS);
- experience with the application of biomarkers has highlighted the need to validate methods with a broader range of species occupying different locations in food webs and reflecting a wide range of lifecycles and feeding mechanisms;
- the advent of genomic and proteomic approaches now presents the possibility of identifying changing patterns of gene and protein expression that may allow more effective identification of specific pollutant threats. Furthermore, such studies provide mechanistic insights that have relevance to the ontogeny of disease in humans;
- the global spread of infectious diseases, and the increasing recognition of the presence of bacteria, fungi and viruses in the marine environment clearly point to the value of biomarker approaches for assessing exposure to microbial threats and in the provision of early warning of the risk of the disease in biota and humans;
- many of the biomarkers used in environmental risk assessments are identical to those used in human risk assessment. This provides a potential means of linking environmental conditions with human health and well-being.

Further, there is a need in environmental studies to use multiple biomarkers in multiple species that are considered key components of marine ecosystems. To accomplish this, it will be important for scientists and other stakeholders to collectively choose model organisms that are good sentinel species, good keystone species, occupy key environmental niches that represent different trophic levels and different types of environments (estuarian, benthic, etc.) and which ideally are also amenable to genetic, physiological and other studies. Developing consensus on such model organisms would be a stepping stone to many different types of studies that could then link with each other to more effectively share information. Such common model species could be the basis of integrated monitoring programs, development and application of genomics tools and biomarkers, and development and application of these species as mechanistic models.

An example of a good model species currently under development is the killifish (*Fundulus heteroclitus*), which is a marine estuarian euryhaline teleost fish. It occupies a key position in the estuarian food web, and its health status at the individual and population level is highly representative of the condition of its food web and ecological niche. Thus it serves as both a keystone species and a sentinel species. Interestingly, its ability to rapidly adapt to changes in salinity is a result of complex physiological processes that are highly representative of several human physiological processes. Thus, it also serves as an excellent model for studying the human disease cystic fibrosis, for example, since both the human disease and killifish salt regulation are dependent on the same protein, CFTR. Genomics tools are now being developed (sequencing of the genome and expressed mRNA genome, development of DNA microarrays, proteomics analyses, etc.) for killifish that will make this an excellent model for environmental monitoring, physiological processes, and responses to multiple stressors. Careful selection of other key species using similar criteria would coalesce the scientific community around common models that could then be developed more rapidly and effectively than an ad hoc selection by individuals. Moreover, this would allow a single database to integrate many different layers of information from a wide variety of studies, as well as providing more robust spatial and temporal analyses.

2.2. Naturally occurring toxins

In addition to synthesized organic compounds, such as chlorinated hydrocarbon pesticides, PCBs, PAHs, and pharmaceutical wastes, potentially toxic metals that are greatly mobilized by man through mining and industrial activities, and radionuclides emanating from the nuclear fuel cycle and from weapons testing, there are other naturally occurring compounds that are highly toxic and that can affect both resident marine animals and human consumers of seafood. These include a broad array of toxins produced by marine organisms, but by far the compounds that have elicited the greatest public health consequences are those produced by harmful algal blooms (Landsberg, 2002). Compounds produced by a diverse microflora, but most particularly involving certain species of dinoflagellates and diatoms, can be accumulated by herbivores (e.g., shellfish) or fish and passed on to man. Massive fish kills have occasionally resulted, in addition to sick humans. Reported incidents of harmful algal blooms have increased markedly in recent years, and this is not simply due to increased vigilance of coastal waters (Smayda, 1990; Hallegraeff, 1993). The eutrophication caused by excessive nutrients introduced into coastal waters, frequently attributable to sewage treatment plant effluent as well as riverine input from increasingly fertilized watersheds, has greatly increased worldwide and has probably contributed to more frequent and prolonged harmful algal blooms (Anderson *et al*., 2002). Some environmental factors have been identified that can trigger certain species to produce toxins, but there is still considerable uncertainty regarding this issue and no generalizations appear possible at this time. Reducing the magnitude of these harmful algal blooms and their toxicological consequences would appear to be tied to reducing nutrient discharge into coastal ecosystems, particularly in poorly flushed bodies of water.

2.3. Complex toxicant problems and possible resolution

A significant issue for all environmental and human health studies is the problem of multiple stressors. One example of this is the complex mixture of environmental contaminants that most ecosystems and most humans encounter. Most studies focus on a single stressor such as a chemical contaminant, pathogen or natural toxin. However, many recent studies using binary combinations of agents have demonstrated that they can interact additively, synergistically or antagonistically. We currently have little knowledge or predictive power to determine how two or more environmental agents may interact to lead to environmental or human health impacts. Yet there are examples where such interactions can lead to "tipping points" where significant effects occur that are poorly predicted by information of each agent in isolation. New studies should focus on those complex interactions that are either most likely to occur and/or have the greatest potential, based on current knowledge, to lead to non-additive interactions. Important mechanistic studies need to be conducted that will evaluate how, and to what extent, whole classes of chemicals are likely to interact with each other in order to develop a greater predictive capability for evaluating risks of organisms, including humans, to environmental contaminants. This will be even more important as emerging contaminants begin to build up in coastal waters, including new synthetic chemicals from industry, pharmaceuticals, etc., and emerging pathogens of concern.

There is a well-recognized continuous and increasing introduction of new contaminants into marine ecosystems, contaminants for which we often have no information on structure, persistence in the environment, reactivity for different environmental matrices, or biological effects (CIESM, 2004). While chemical analyses can help determine the exposure concentrations of xenobiotics, this type of analysis is limited because only known substances are analyzed, the interaction of mixtures of compounds is not taken into account, and currently the biological effects of these mixtures cannot be predicted. Moreover numerous organic contaminants can be metabolized and transformed into other compounds that can sometimes be more toxic than parent compounds (Schwarzenbach *et al*., 2003). The multi-contaminant (multi-sources, multi-classes) character combined with the temporal and spatial variability of their abundance, as well as complicating natural interactive forces (varying salinity, temperature, sunlight, etc.) makes more and more complex the evaluation of the contamination of environmental systems and the risk assessment procedure for the marine environment and for human health. In some cases, there may also be significant toxicological interactions between co-occurring toxins produced by blooms of some algae and these synthetic organic compounds or metals, but this is largely a matter of speculation at this time. Some progress may come with the development of screening methodology with no *a priori* selection of chemicals linking toxicity bioassays and chemical analyses. The new possibilities provided by the recent development in bioassay technology lead to the availability of a battery of specific bioassays able to determine the *in vitro* CMR (carcinogenic, mutagenic, reprotoxic) potency of chemical mixtures. By developing a “Toxicity Identification Evaluation” (TIE) type approach combining fractionation of total extracts, bioassays and chemical characterization of the toxic fraction, it may be possible to identify the toxic compounds present in the natural environment and in products related to human consumption.

Many of the new “emerging” contaminants are associated with discarded pharmaceutical chemicals, many of which enter coastal water through sewage treatment plants. Measured concentrations fluctuate between low ng l^{-1} to tens of $\mu g\ l^{-1}$ levels, depending on the compound, location, and season. Most studies have assessed concentrations in the dissolved phase, but the particulate phase (phytoplankton, suspended abiotic particles) may also be important and may serve to introduce these substances into marine animals even more effectively than the aqueous phase (Fisher and Reinfelder, 1995). The bioaccumulation of most of these compounds in aquatic organisms, biological and abiotic breakdown products, and toxicity are just starting to be examined and need to be explored much more fully, as these could influence their impact on human health as well as the health of resident marine animals. Regarding antibiotics, bioaccumulation properties of some are known (e.g., in relation with their use in aquaculture) and there are EU norms fixing their maximum levels for products consumed by humans.

Genomics and proteomics represent two new experimental tools that can provide powerful information about the biological and toxicological responses of organisms, including humans, to environmental factors including chemicals of concern, pathogens, and other factors. Genomics provides the most sensitive tool for investigating very low level biological responses, and its ability to “fingerprint” unique responses to individual factors provides a means of both investigating a mechanism of action and using these tools for environmental monitoring and toxicant detection. Genomics can also be used to investigate complex and poorly understood phenomena such as non-monotonic dose responses including hormetic effects, potentially non-additive interactions of multiple stressors, and population shifts in response that are not obvious on the individual level. Proteomics provides a means of developing and validating powerful protein level biomarkers of exposure, susceptibility and effect that, in combination, can be used to assess complex environmental responses including changes in human response that presage subsequent health impacts. Collectively these tools rely on genomics information about the individual species under investigation. Thus, to most effectively apply genomics and proteomics to the marine environment it will be necessary to develop appropriate genomic information about model species under investigation.

3. MICROBIAL PATHOGENS IN COASTAL MARINE ENVIRONMENTS

The contributions of Ingrid Brettar and Carlos A. Guzman to this section are gratefully acknowledged.

3.1. Pathogenic microorganisms of relevance to human health in the marine environment

Most human pathogens are found in coastal marine environments and not in open ocean habitats. This is commonly attributed to human activities on the coastline. Bacteria are the best studied of all known pathogens of marine origin (Belkin and Colwell, 2006). We know that only a very limited number of bacterial taxa contain pathogenic species (*Gammaproteobacteria*, *Epsilonproteobacteria*, *Firmicutes* and *Actinobacteria;* Brettar *et al*., this volume). Some of these bacteria are well known as indigenous marine bacteria, e.g., the *Vibrio* species, or freshwater bacteria from continental inputs, e.g., *Aeromonas* species (Monfort and Baleux, 1991), whereas others like *Salmonella* sp. and *Helicobacter pylori* are of allochthonous origin (Carbone *et al*., 2005). In addition to the well known pathogenic species or strains, "new species or strains" have to be considered as potential health threats when cases of infections start increasing (e.g., infections by *Mycobacterium marinum*, *Erysipelothrix rhusiopathiae*; Oliver, 2005) or when potentially pathogenic bacteria are detected in high numbers in marine coastal waters (e.g., *Arcobacter* spp.; Maugeri *et al*., 2004). Furthermore, the occurrence of pathogenic bacteria equipped with new virulence or antibiotic resistance genes through horizontal gene transfer (e.g., by bacteriophage transduction), or hybrid organisms generated from two genomes of non-pathogenic bacterial strains (Bisharat *et al*., 2005) can result in highly virulent bacteria representing a sudden and unexpected threat for human health. Thus, we have to take into account the threat by emergence and re-emergence on the species, subspecies, and clonal level, which is a major challenge for the provision of adequate detection tools and prevention measures.

The discovery of pathogenic viruses in marine environments is a relatively recent event with the first detection of *Coxsackievirus* in 1947 by Dalldorf and Sickles (for a review see Griffin *et al*., 2003). The four major families of pathogenic viruses found are *Adenoviridae*, *Caliciviridae*, *Picornaviridae* and *Reoviridae* (Brettar *et al*., this volume). These families comprise double stranded (ds) DNA, single stranded (ss) RNA and ds RNA viruses. All known pathogenic viruses that pose a significant health threat in the marine environment are transmitted via the fecal-oral route (hence their name as "enteric viruses") and are considered to be of allochthonous origin.

In addition to viruses, three genera of zoonotic protozoa, *Giardia*, *Cryptosporidium* and *Toxoplasma*, are detected in coastal waters (Fayer *et al*., 2004), sediments and shellfish. The origin of the protozoa is fecal matter of human or (mostly domestic) animals. Protozoa survive well as cysts that are small, buoyant, and resistant to most environmental influences, even to disinfectant, and remain infective in moist environments for long periods (up to a year); thus protozoan cysts are much more resistant to environmental stress than viruses and bacteria (Nasser *et al*., 2003). The occurrence of terrestrial protozoan species like *Cryptosporidium parvum* in shellfish indicates their land origin. Infection by these protozoa in the marine environment is observed for a large set of marine mammals.

Pathogens found in the marine environment are responsible for a broad spectrum of acute and chronic human diseases such as gastroenteritis, ocular and respiratory infections, hepatitis, myocarditis, meningitis, and neural paralysis. The origin of most marine pathogens is feces, with only a very few "autochthonous bacterial pathogens" (e.g., vibrios) able to grow in the marine coastal environment.

3.2. Ecology of marine pathogens

Survival of human pathogens in the marine environment is controlled by ecological mechanisms and environmental factors, such as grazing, solar radiation and temperature, organic matter availability, and suspended particle load. Infectivity might also depend on the physiological state and ecological fate of the pathogen and of the specific species that is infected. Concentrations of pathogens are strongly influenced by sewage discharge, ship traffic, and meteorological events like heavy rainfall. At present, despite a growing body of work in the field, there is still an inadequate understanding of the factors that affect the amount, survival, activity and infectivity of pathogens in marine systems.

Viruses and parasitic protozoa (in cyst form) are not considered capable of physiological responses to environmental stresses and can be regarded as almost inert biological particles in this respect. In contrast, bacteria are capable of activating different mechanisms to respond to such stresses and to survive in sub-optimal environmental conditions; a good example would be endospore formation by some gram-positive bacteria (e.g., *Clostridium*). In non-sporulating bacteria, cells arriving at the stationary phase of growth activate genetic cascades that enable an increased resistance to a number of stresses such as high or low temperature, increased salinity, osmotic shock and other environmental stressors (Jenkins *et al*., 1990; Ostling *et al*., 1993). Another adaptation mechanism more recently discovered to hostile environmental conditions is known as the Viable But Non Culturable (VBNC) state (Colwell and Huq, 1994; Lipp *et al*., 2002; Xu *et al*., 1982). A stressed bacterial cell that grows normally on culture media loses this capacity by entering the VBNC state, but can stay physiologically active and retain its virulence. The VBNC state was described for numerous allochthonous and autochthonous pathogenic bacteria. For autochtonous bacteria, such as *Vibrio*, the response would be an adaptation of the cell, for example in cold winter conditions. However, for an allochtonous pathogen such as *Salmonella*, the VBNC state can be considered an artifact of cellular degradation caused by environmental stresses that may lead to cellular lysis (Monfort and Baleux, 1994).

3.3. Ecological mechanisms supporting survival of pathogens in the marine environment

Organic nutrients improve the survival and growth conditions of bacteria in coastal waters. They can derive from algal growth or the land, introduced by river discharge, sewage, and runoff. The highest concentrations of organic nutrients are often found on the surface of organisms like algae or copepods. Attachment to these surfaces therefore provides a favorable environment for bacteria, including pathogenic bacteria like members of *Vibrio* genus (Lipp *et al*., 2002).

Attachment of microorganisms to particles in general increases survival and infectivity of bacteria and viruses (Brettar and Höfle, 1992; Griffin *et al*., 2003; Maugeri *et al*., 2004). For bacteria, improved nutrient conditions, shelter from grazing and UV irradiation improve the survival or growth conditions. For viruses, shelter from UV irradiation supports survival and infectivity. For protozoa, particle attachment seems to have little effect on survival in water. For all microorganisms, particles may serve as a vehicle for transfer to the sediment.

Attachment to plankton is of special relevance for the survival and growth of pathogenic bacteria in the marine environment. Attachment can occur to zooplankton and phytoplankton that both provide increased availability of organic nutrients and shelter from UV and grazing. For vibrios and other bacteria including fecal indicators, attachment to zooplankton increases survival, growth and distribution (via transport by the animal host and/or its feces) (Baffone *et al*., 2006; Maugeri *et al*., 2004; Signoretto *et al*., 2004).

Interactions with bivalves and other invertebrates provide protective and growth-promoting conditions for some pathogenic marine bacteria (e.g., vibrios) similar to attachment to plankton (Cavallo and Stabili, 2002). The resistance of some pathogenic bacteria and viruses to depuration procedures of edible bivalves is a further reason for the world-wide incidence of seafood borne diseases. Factors such as bacterial surface ligands, soluble hemolymph components, and the ability of some bacteria to activate distinct stress-signalling pathways involved in the hemocyte response are important in determining the bacterial fate within a bivalve host (Canesi *et al*., 2005; Pruzzo *et al*., 2005a). Interestingly, recent evidence shows that colonization ligands used by *V. cholerae* to interact with chitin-containing surfaces can be the same as those required for intestinal colonization (Kirn *et al*., 2005; Zampini *et al*., 2003). This observation points to a common link between the persistence of some potentially pathogenic bacteria in the environment and infection of the human host and suggests that virulence mechanisms of bacteria having environmental reservoirs may reflect adaptive mechanisms to the environment.

All pathogenic microorganisms show increased concentrations, prolonged survival and infectivity in sediments, especially surficial sediment (Fayer *et al*., 2004; Griffin *et al*., 2003; Brettar and Höfle, 1992). Thus, sediments that are resuspended by storms and ship traffic may introduce pathogens into the water column, particularly in lagoons and harbors. Reduced grazing and protection from UV irradiation could be important for the improved survival conditions of microorganisms, but further studies of the environmental factors that affect the survival and activity of pathogens in sediments are clearly needed.

3.4. Mechanisms reducing survival of pathogens in the marine environment

All pathogenic microorganisms are subject to grazing in the marine environment to some extent. Most relevant grazers are flagellates, ciliates, zooplankton such as cladocerans, filter-feeding bivalve molluscs, ascidians, polychaetes, and sponges. Grazing involves ingestion and digestion of the microorganisms. Bacteria are to some extent eliminated by grazing. Viruses are the least affected by grazing due to their small size.

Bivalves often exhibit a strong enrichment of pathogenic bacteria and viruses relative to the surrounding seawater (Miossec *et al*., 2000; Muniain-Mujika *et al*., 2003). Most gram-negative marine bacteria are considered to be well digested by bivalves (Birkbeck and McHenery, 1982), but many pathogenic bacteria, especially vibrios, are able to survive and even multiply within the bivalve gut (Charles *et al*., 1992; Pruzzo *et al*., 2005a).

UV radiation is able to destroy viruses and bacteria. Since UV radiation decreases with water depth and turbidity, bacteria and viruses in deeper water layers and/or particle-rich water are less stressed by UV radiation than in clear surface water. As well most pathogenic bacteria are more abundant at higher water temperature which promotes faster growth. With decreasing temperature, an increased fraction turns into a VBNC state (Huq *et al*., 2000) and the infectivity and survival rate may decrease. By contrast, viruses survive longer and display a higher rate of infectivity at lower temperature. Protozoa survive for a long time (as cysts up to a year or even longer) regardless of temperature (Fayer *et al*., 2004).

Bacteriophages can infect bacteria and lead ultimately to lysis and thus elimination of the bacterial cells. Bacteria occurring in the marine environment can either be infected before they enter the marine environment (e.g. in the intestine of a land animal, or in the sewage treatment facility) or be infected in the marine environment. Due to their specificity, the host spectrum of a specific phage is very restricted, usually to a specific bacterial species. Since the probability to be infected by a virus of the right specificity is dependent on the abundance/density of a bacterial host, bacteria with a high abundance in the coastal environment - such as vibrios or *Aeromonas* sp. - are much more likely to be infected and killed by viruses in the marine environment (Jiang *et al*., 2003). For bacteria unable to grow in the marine environment, infection by viruses of marine origin is unlikely; by contrast the presence of bacteriophages specific for enterobacteria can be used as a tracer for fecal contamination in shellfish or in coastal water (Dore *et al*., 2000).

3.5. Current hygienic problems in coastal environments

The main sources and transmission mechanisms of microbial contamination in coastal marine environments are sewage, run-off, floods, groundwater seepage, and river discharge. In specific areas, ship traffic, ballast water, and bathing represent additional sources. Well-treated sewage is intended to contain low numbers of bacteria, viruses and protozoa and the load of pathogenic microorganisms increases with decreasing treatment procedures/quality. Major problems arise during heavy rainfalls, storm events or floods that flush untreated or improperly treated sewage into rivers and coastal water, often leading to increased load of pathogens in coastal waters and increased incidence of disease (Miossec *et al*., 2000). It is noteworthy that treated and disinfected wastewater may still have a high load of pathogenic organisms, averaging 30 - 80% of that in untreated sewage (the lowest values for bacterial pathogens, the highest for protozoan cysts) (Harwood *et al*., 2005). Disposal of sewage sludge in agricultural areas, rivers and coastal regions can be another important source of pathogenic microorganisms.

Run-off carries pathogenic microorganisms from soils and urban surfaces and, depending on the strength of the rainfall, overloaded sewers, rain water collectors and sewage treatment facilities to rivers and coastal water. Depending on the season, this might be a threat to human health via contaminated seafood or seawater. Microbiologically contaminated groundwater may seep directly into coastal water or be transferred via river water. For viruses high transfer rates from groundwater aquifers, especially in limestone, to coastal water have been observed. Major contaminators of groundwater are households with septic tanks, livestock, and leaking sewage pipes. Rivers often carry high loads of pathogenic microorganisms, especially following rainfall events, to coastal waters.

In summary, feces of humans and domestic animals are major sources of pathogenic microorganisms entering coastal marine environments. The complete transport of feces to sewage treatment plants is difficult to achieve, especially during periods of heavy rain. Additionally, pathogenic bacteria, viruses and protozoa are somewhat reduced but not eliminated in sewage treatment plants (Harwood *et al*., 2005); those deposited in the sewage sludge are another source of contamination unless effective further treatment was achieved.

Furthermore, man-made eutrophication of coastal waters due to the addition of inorganic nutrients increases the production of algal biomass which in turn improves the survival of pathogenic bacteria by increasing the availability of organic nutrients and particles; the increasing particle load can also enhance the survival of pathogenic viruses. Global warming results in increased temperature of coastal waters, and this would also enhance survival of pathogenic bacteria but may diminish survival of viruses.

3.6. Emerging threats and potential mechanisms to increase virulence of microorganisms

Highly pathogenic microorganisms can either be directly introduced into the marine environment, or increase their pathogenicity in the marine environment. Major sources for introduction are sewage, with sewage derived from hospitals, and inadequately treated sewage especially from large urban areas representing a great threat. Additionally, ballast water has to be taken into account - not only for toxic algal blooms but also for the transfer of highly pathogenic microorganisms (Peperzak, 2005).

Mechanisms for increasing the pathogenicity for bacteria are i) horizontal gene transfer in aquatic breeding grounds, such as fish farms and overloaded sewage treatment plants, that allows acquisition of genes responsible for virulence or antibiotic resistance from other (usually related) bacteria or via bacteriophages, or - as recently shown - ii) formation of a hybrid highly pathogenic organism generated from two genomes of non-pathogenic bacterial strains (Bisharat *et al*., 2005). Horizontal gene transfer is more likely with increasing density of a specific pathogenic bacterial species or genus, e.g. more likely for vibrios than for enterobacteria and for sites of high concentration, such as gills of bivalves (Miller, 2001). Bacteriophages can serve as vehicles for nucleic acids of bacterial origin (genomic or plasmid DNA) that can lead to transduction of bacteria. A good example is the cholera toxin that is encoded by a lysogenic bacteriophage (Faruque *et al*., 1998). Chitinous surfaces are ubiquitous in marine environments on living crustaceans or their cast exoskeletons; it has been recently shown that *V. cholerae* interactions with such surfaces induce regulatory circuits that control transformation, thus providing bacteria another genetic mechanism to acquire new virulence or antibiotic resistance genes (Meibon *et al*., 2005).

The potential for contracting an infectious disease depends on different factors, including host susceptibility, the degree of exposure to the pathogens and the virulence of the agent. The increasing number of both aging and immunocompromised individuals, who are most susceptible to infections, emphasize the need for setting up control measures that are directed towards microorganisms with low virulence potential, such as *Aeromonas* spp.

It is now recognized that health issues, including those related to water borne diseases, are no longer simply a local problem but encompass global issues (Colwell, 2004). Natural events, which are now known to occur on a global scale, the worldwide moving of people (e.g., business, tourism) and goods, and human activities are largely responsible for the greatly enhanced global transport of microorganisms, including well known pathogens and novel pathogens such as SARS and extremely drug-resistant strains like *Mycobacterium tuberculosis* type XDR.

3.7. Possible solutions to improve the hygienic conditions of coastal seawater

Currently, hygienic quality and potential health risk from exposure to seawater and consumption of contaminated seafood are assessed by culturing indicator bacteria. This classical microbiological methodology relies on such methods as plate counts (CFU) of coliforms or plaque forming units (PFU). There are some serious deficiencies with these measurements there is no significant correlation between their outcome and the abundance of many microbial pathogens of non-fecal origin, nor do they provide valid identification of the pathogen. For this objective state-of-the-art molecular detection methodology has to be considered. Advanced methodology for environmental detection of microorganisms depends on: i) the type of microorganism, ii) the level of taxonomic resolution to be achieved, iii) the detection limit to be reached, and iv) the cost and time necessary to analyze samples. The approach now used widely is to analyze nucleic acids, extracted directly out of seawater, with a suite of molecular methods ranging from PCR and DNA array-based techniques to immuno-capturing and fluorescence *in situ* hybridization (FISH). Applying quantitative PCR techniques such as real-time PCR has enabled quantifying the pathogenic microorganisms in seawater without culturing them (Fey *et al*., 2004; González-Escalona *et al*., 2006). These advanced methods require considerable expertise and facilities and as yet are not routinely used for seawater analysis. There is clearly room for technological improvement, standardization, validation and automation of the molecular detection technology, particularly as it relates to pathogen detection in coastal waters. Validation of molecular detection methods for the environmental detection of microorganisms is also recommended for quality control of drinking water (OECD, 2003).

3.8. Interrupting the fecal-oral infection route by integrated coastal management

Transfer or storage of pathogens in sewage effluent seems to be a task that cannot be done without occasional high contamination of surface, ground water and coastal water. A solution to the risk of storage and transport would be a rapid on site extinction of these pathogens. Efficient procedures to reduce the load of pathogens (bacteria, viruses, protozoa, helminths) in human and animal waste in a short period of time (one to a few days) are those that include a step with heating of the waste, such as composting (>60°C, due to microbial heating) and the thermophilic production of biogas by methanogenesis (> 50°C). Critical to these procedures is that they reach high temperatures (above 50°C) for a sustained period (one to a few days). These hygienic measures for waste treatment are well established and could contribute to a healthier coastline; they are also profitable if the production of biogas is included in the treatment (Verstraete *et al*., 2002; Erickson *et al*., 2004).

A reduction of the transmission of zoonotic protozoa from pastures to river and coastal water can be most effectively achieved by "landscape solutions", i.e. riparian buffer strips and reforestation, measures that reduce erosion and rapid run off, and improve the "filtering capacity" of the landscape. These measures will counteract in the same way non-point nutrient sources such as the transfer of nutrients (especially N and P) from the land to the ocean and thus reduce eutrophication of coastal water.

Generally, steps taken to reduce coastal eutrophication from point sources and non-point sources will likely reduce autochthonous pathogenic bacteria, such as vibrios, by removing a major source of organic nutrients for these bacteria (Mourino-Perez *et al*., 2003). Additionally, phytoplankton is the food for zooplankton - another major site for vibrio growth and dispersal. Thus, the hygienic treatment of waste and waste water, together with measures to reduce transfer of pathogens and nutrients from point and non-point sources, could provide the basis for an integrated coastal management scheme that could greatly reduce the occurrence and growth of pathogens in coastal environments and ensure safe bathing and seafood consumption.

3.9. Surveillance, epidemiology and integrated monitoring of marine-borne infectious diseases

Epidemiological studies of marine infectious diseases are lacking for almost all important aspects including the etiological agent, transmission routes, links between virulence properties and illness severity, number of cases and size of population exposed, etc. Therefore, a critical need and priority in the study and control of seawater-borne diseases is an epidemiologically structured network of surveillance based on contributions by public and private institutions, physicians and international health organizations.

Current monitoring of water quality in terms of pathogens is generally performed on a regular basis, mostly due to legal obligations. For example, at shellfish farming sites, monitoring of fecal indicators is usually done weekly. At bathing sites, this monitoring is often done weekly or bi-weekly during summer. However, the dynamics of pathogens in seawater, while still not well understood, suggests that marine bacterioplankton can rapidly respond to environmental changes within hours. Therefore, current sampling schemes are probably inadequate to predict human health threats originating from pathogens. An integrated monitoring approach appears to be the most promising solution to effectively protect human health considering all the parameters and forces influencing the presence, survival, activity and the actual infective dose of pathogens in seawater.

Integrated monitoring should combine all information available for a specific region, including GIS-based landscape data of the physical shape of the coastline and the underlying water body, physical, chemical and biological parameters (e.g., temperature, salinity, chlorophyll) integrated with dynamic hydrographical and meteorological data. To address public health issues, current and archived hygienic water quality data, such as indicator counts for *E. coli* and/or other indicator organisms, should be integrated and modeled in a dynamic way to understand the occurrence and spread of seawater-borne pathogens along the coast. More refined molecular data sets on the direct assessment of abundances of specific pathogenic viruses, bacteria and protozoa, should be added and, if their seasonal and spatial behavior has been assessed, can be modeled and integrated for the dynamic forecasting of health threats due to seafood consumption or bathing. Some particular sites (polluted or non polluted, closed or open bay) could then be used as test sites to validate dynamic monitoring models and their utility as early warning tool to prevent human infections. Other sites could be chosen for monitoring some pathogens in a continuous and long-term manner, to generate a sentinel network for the prevention of outbreaks of infectious diseases. These data should be integrated with environmental data specific to climate change so as to develop a long-term baseline for the assessment of health effects due to climate change.

An integrated monitoring approach could provide the basis for an early warning system capable of alerts regarding public health emergencies. Towards this end, remote sensing technologies with satellites could provide an important contribution with their dual capability of performing continuous environmental monitoring with coverage of large areas and acquiring/ transmitting data in real time. The establishment of possible correlations between specific parameters and the quantity and distribution of pathogens would be the basis for an early warning system on water-related health risks.

3.10. General conclusions and future research needs regarding pathogens

There is a rather limited knowledge on the microbiological principles governing the prevalence and pathogenesis of human microbial pathogens in the marine environment. The reasons for this lack of knowledge are that: i) the precise detection, identification and quantification of microorganisms in water are difficult and only possible with a combination of classical and molecular methods; ii) the virulence of waterborne pathogens varies with environmental conditions and the type of pathogen; and iii) the transmission of waterborne infections to humans via seawater is a complex process depending on the type of pathogen, infectious dose, immune status of the human and a multitude of other factors.

We foresee the following future research needs:

- development, validation and standardization of rapid detection methods for the most relevant pathogenic viruses and bacteria in seawater and seafood;
- abundance studies of major pathogens in the marine environment to elucidate their biogeography and their environmental control factors;
- thorough investigation of emerging marine pathogens including genomics, ecophysiology and host-pathogen-interaction;

- studies on the survival of pathogenic microorganisms in marine environments;
- studies on sanitation and sewage treatment conditions with respect to elimination / inactivation of pathogenic bacteria, viruses and protozoa;
- study of the epidemiology of seawater-borne and seafood-borne diseases originating from human pathogens of the marine environment.

4. CONTRIBUTION OF MARINE MONITORING PROGRAMS TO HUMAN HEALTH ISSUES

In order to protect the health of seafood consumers, many countries and international regulatory bodies have set guidelines for the maximum permissible levels of some well-known toxic contaminants (metals, chlorinated hydrocarbons, PAHs, etc.), bio-toxins and pathogens in seafood (e.g., European Commision, 2001). It is particularly important to monitor levels of key contaminants and pathogens in the coastal zone, where most fishery and farming activities are carried out and where contamination is most acute. Shellfish may harbor elevated contaminant and pathogen levels and serve as conduits for their passage to human consumers; they are therefore commonly monitored, as are coastal bathing waters during summer months for fecal indicator bacteria.

Monitoring contaminant levels in seafood is presently the only way to comply with seafood safety guidelines established by regulatory bodies. These measurements are also used to evaluate the extent to which remediation activities or pollutant reduction activities are effective. Monitoring can also reveal information for better understanding processes relating to the bioaccumulation and effects of contaminants in ecosystems (CIESM, 2002a,c). This has long been recognized, and research over the past two decades has attempted to combine field monitoring efforts with lab and field experimentation to provide explanations of field observations.

Biological indicators, or "sentinel species", have been analyzed in monitoring program to determine baseline levels of key contaminants and assess their spatial and temporal trends in coastal regions. The choice of such indicator organisms is driven by two main constraints: relevance and feasibility. Probably the most widely applied bioindicator organism in the world is the mussel *Mytilus edulis*, or in the Mediterranean *Mytilus galloprovincialis*. This mussel has many attributes that make it suitable for assessing contaminant levels in coastal waters: it is sedentary, fairly ubiquitous, generally resistant to high levels of most commonly occurring contaminants (enabling it to inhabit heavily contaminated regions), and it accumulates contaminants from both the aqueous phase and from food such that tissue concentrations of contaminants reflect an integrated history of what the mussel experienced over extended periods of time. Perhaps most important, tissue concentrations of contaminants in the mussel clearly reflect the biologically available fraction of that contaminant. However, interpretation of mussel tissue data can be more complicated than that of plant material, since mussels eat food and can obtain some of their contaminants very appreciably from their diet (Wang and Fisher, 1997) in addition to the aqueous phase, which is the only source for such bioindicators as macroalgae. The advantages and limitations of using different biological indicators have been reviewed (Phillips, 1980). Regardless of which species are used, our understanding of the factors governing biological concentrations of contaminants often evolves over time, and so monitoring strategies must incorporate relevant experimental research to keep pace.

To ensure that meaningful spatial and temporal trends in contamination can be discerned using bioindicator organisms, every attempt should be made to use a single indicator species, since significant differences in contaminant bioaccumulation or degradation commonly exist between species. When the desired sentinel species is not naturally present in some regions, as is the case for the Mediterranean mussel (*Mytilus galloprovincialis*), it is possible to transplant bio-indicator organisms in caged devices, which is now a standard procedure (Andral *et al*., 2004). The transplantation/caging technique also helps minimize biological variability as it relies on the use of a calibrated batch of mussels from one single population deployed and then sampled at the same time in the different monitoring stations.

Passive samplers may hold promise to help understand the availability of dissolved, but not dietary, contaminants for marine organisms; they might therefore be useful for those contaminants for which diet is a relatively unimportant source term. Most use specific ligands and membranes with high affinities for certain classes of contaminants. These devices include the Semi-Permeable Membrane Device (SPMD) for hydrophobic compounds, the Polar Organic Compounds Integrative Sampler (POCIS system) for hydrophilic compounds, and Diffusion Gradient Thin Film (DGT) for metals. Once calibrated and validated, these tools could simplify logistics and sampling effort over time.

In addition to the problems associated with monitoring for xenobiotic organic compounds about whose environmental fate and bioaccumulation we still know relatively little, there are further complications involving the transformation of parent compounds to various degradation products, some of which are more toxic than the parent, such as some of the polycyclic aromatic hydrocarbons. Given the complications associated with monitoring for numerous contaminants, often co-occurring and often poorly understood, one approach that is increasingly adopted is to develop screening protocols that link chemical analyses with toxicity bioassays. The new possibilities provided by recent developments in bioassay technology lead to the availability of a battery of specific bioassays able to determine the *in vitro* CMR (carcinogenic, mutagenic, reprotoxic) potency of chemical mixtures. By developing a “Toxicity Identification Evaluation” (TIE) type approach combining fractionation of total extracts, bioassays and chemical characterization of the toxic fraction, it could be possible to identify the toxic compounds present in coastal regions and seafood.

As noted above, monitoring coastal regions should also consider discerning distributions of pathogenic microorganisms as well as chemical contaminants. In this context, different preservation procedures, such as deep freezing, can help archive samples in an appropriate format; given the uncertainties in this field at present and the current limitations of current methodologies, archiving samples for future analysis will likely prove to be of great importance. Archive samples accompanying current monitoring efforts would enable the establishment of an appropriate baseline for marine ecosystems, provide the possibility of tracking major contamination accidents or outbreaks of seawater-borne infections, and allow for future analyses of samples with new methods, new standardization or validation procedures. Ideally such an archiving program would be done on a large regional scale that would harmonize national efforts and establish common rules and procedures.

Satellite images are now broadly available to the scientific community: altimetry data, sea surface temperature and sea color (from which chlorophyll and suspended matter contents can be derived) are regularly provided with high resolution at the regional scale. Although satellite sensors can not measure dissolved contaminants or pathogens, these large-scale, high-frequency oceanographic data sets, coupled with circulation modeling, could guide monitoring strategies with regard to selecting sampling stations on appropriate spatial and temporal scales. Satellite surveys have already begun to evaluate water management strategies in France and are generally well suited for detecting plumes of visible contamination, such as oil spills and sewage treatment effluent, as well as chlorophyll levels in surface waters. The latter can provide evidence for the evolution of algal blooms following eutrophication, and all the attendant issues associated with these blooms. In time, it is expected that additional photosynthetic pigments can be detected, which may help identify blooms of potentially toxic phytoplankton (e.g., some dinoflagellates) that can impact the health and harvestability of some marine animals, including fish and shellfish. Satellites can also provide synoptic assessments of land use patterns in watersheds that may affect coastal ecosystems.

Such data should be stored in databases that are accessible by scientists and governmental agencies involved in monitoring efforts. These developments shall be considered in the European program GMES (Global Monitoring for Environment and Security). GMES is a joint initiative from the European Commission and the European Space Agency for the purpose of providing, on a sustained basis, reliable and timely services related to environmental and security issues in support of public policy makers’ needs. It will cover global, national and regional issues relating to climate, environment management and civil security. GMES aims to coordinate existing systems, produce services of guaranteed validity, and ensure their future continuity. It will stimulate capacity growth through the creation of new observation sensors and a wide variety of added-value services. GMES will be based on a comprehensive earth observing system, including different types of data (space, airborne, *in situ*), efficient data management and information sharing, and organized services deployable on a European scale.

The first operational services will deploy in 2008. GMES has been declared as the main European contribution to the GEOSS (Global Earth Observation System of Systems) whose implementation plan was signed in February 2005 in Brussels by 60 countries and 40 Organizations. Initial services were defined to establish the foundations of GMES and include the marine and coastal environment (including pollution, oil spills, water quality).

The workshop allowed a first attempt for scientists to identify specific needs for a reference data base on coastal areas (Table 2).

Table 2. Specific needs for satellite data for understanding coastal contamination.

Application	**Spatial resolution**	**Time frequency**	**Accuracy**	**Need and priority**
Sea Data				
Coastline	10 m	Once a year May be more for change detection	3 m	High
Bottom color	10 m	Once a year or on request	low	High Sediment accurate location Algae location Need of separation between mineral and vegetation
Classification of mineral/biological matter		Once a month or on request		High importance just to separate biological from mineral matter
Water color White phenomenon Green Red	5 -10 m	During summer		High for particular phenomenon
Chlorophyll measurements	10-30 m	Once a month or on request	mg/m^3	Only if there is a continuous spectrum from blue to NIR (1.2 µm) Useful obviously for microbiologist
Suspended matter	10-30 m	Once a month or on request	mg/m^3	High Particles distribution
Turbidity	10-30 m	Once a month or on request		Important regarding the illumination of the bottom by the sun
Location of sand bank	10-30 m			No
Trace Metals				OK for Mo, Mn, Fe
Coastal currents	50 m	Once a month or on request		Highly important
Local pollution (e.g., oil spill) Sewage Floods events Meteorological events River plumes	10-30 m	On request		Important
Water temperature	50 m	Once a month or on request	One degree resolution at least 0.1 be better	High
Aerosols concentration from sea level up to 500 m.	50 m			High
Land Data				
Land use	10-30m	Once a year		High Catchments area (basin versant) of the Mediterranean Sea
Location of ports, dikes, towns sewage, farms, pipelines, industries	5-10m	Once a year		High
3D Data				
Bathymetry	10-30m	Once a year	No need of a high accuracy	Low
DEM	10-30m	Once a year		High
Connection Bathymetry-DEM	10-30m			High
Hydrographic network				High to Low, depending on the activity
Temporal survey				
For pollution detection and survey	Every 6 hours Daily data Weekly data			
What geographical surface?	10km from the coast offshore			
What kind of phenomenon?	Pollution			

5. Specific Mediterranean issues

The Mediterranean Sea represents a unique marine environment as it is a large semi-enclosed oligotrophic system with its own unique oceanographic properties, including a water temperature that remains ≥ 12ºC, a fact that will affect growth and survival of pathogens, degradation of organic compounds, etc. It is surrounded by countries with greatly varying degrees of industrialization and agricultural development. A key feature of the Mediterranean region is the uneven distribution of expertise in the fields of environment and human health. The emergence of new threats and new technologies to monitor and mitigate impacts highlight the need for building human capacity in these areas, especially in countries where economic challenges are greatest, notably along the southern Mediterranean coast. Areas in which training programs are urgently needed include environmental informatics, integrated environment and human health risk assessment, detection and monitoring of infectious diseases, and in the use of new tools. Training should involve exchange visits among riparian states. A positive outcome would be a transfer of knowledge and increased understanding and effective working relationships among scientists in the region.

There is no doubt that the amount of information available regarding the environment and human health in the Mediterranean region is increasing exponentially, although considerable gaps in our knowledge on a wide range of topics still remain. It is timely to consider synthesizing data around certain themes like contaminant distribution, exposure to infectious diseases, disease outbreaks, etc. It would then be possible to investigate the relationships among a wide range of phenomena and develop integrated strategies to protect environment and human health. There is a need for a new Mediterranean bioinformatics program consisting of databases integrating data from diverse studies in common formats that can be analyzed in a multi-variate manner. Such integration will also require new analytical tools including complex modeling and systems biology approaches that will be useful to other similar large system studies.

Social patterns may also influence the extent to which health-threatening contaminants enter the Mediterranean Sea and affect public health. There are striking increases in local populations during the summer tourist season, for example, during which time more contaminants enter coastal systems through sewage (treated and untreated) and more seafood is consumed. Because there are substantial cultural differences among the human populations surrounding the Mediterranean, different lifestyles and food habits must be carefully examined when addressing public health issues related to seafood consumption. For example, religious restrictions forbid some populations from eating shellfish and almost all harvested or cultivated production is exported to EU countries. Hence, while no direct health concerns may exist for local populations, seafood safety must comply with EU guidelines and regulations. Even desalinated Mediterranean water can serve as a source of drinking water (and associated contaminants) in some areas, and again variations among cultures are pronounced. It is therefore appropriate to use critical pathway analysis, first established for radioactive wastes, to determine the health risks related to specific types of chemical contaminants and pathogens for the most vulnerable groups in the Mediterranean.

Sea change: implications for human health and well-being

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ABSTRACT

Climate change is now widely recognised as being one of the greatest threats to human health and well-being in the Mediterranean region in the coming decades.

Impacts are being superimposed on those associated with point source and diffuse pollution, habitat and biodiversity loss, overfishing and the development of coastal regions for tourism and industrial activity. This is happening at a time when demographic studies are revealing increased population densities in coastal areas, and indeed, growth in the population of the whole region. In this paper, these issues are reviewed and examined in some detail. The options available to environmental managers and policy makers are discussed. In particular, a shift away from chemical monitoring to biological effects studies is advocated, as is the need for a more integrated approach to policy formulation and implementation.

INTRODUCTION

There is widespread recognition that climate change is profoundly affecting life on Earth. Evidence suggests that it will continue to do so for the foreseeable future (May, 2006). A major consequence is that our seas are also changing. As the icecaps and glaciers melt, sea level is rising and ocean currents are altering. One third to a half of anthropogenic CO_2 emissions have been absorbed in the oceans (WBGU, 2006). As this process continues, seawater will become increasingly acidic. Inevitably, these changes in our seas will impact mankind.

The relationship between the status of marine ecosystems and human health is both intimate and complex. The world population continues to grow with 25 to 50% of people living in coastal zones (Bowen *et al*., 2006). As our numbers double over the next 40-50 years, the population of coastal regions will undoubtedly increase still further. Average population density is already 10% higher on the coast than in inland areas and human activities are dramatically reconfiguring the coastal environment. Natural resources are being depleted, natural chemical cycles are being disturbed and enormous quantities of waste are being generated, including toxic chemicals, radiation and heat. It is vital to recognise that anthropogenic pollution of marine ecosystems is superimposed on effects associated with climate change. It is their integrated impact that is leading to changes in biodiversity and damage to ecosystem structure and function, as well as the collapse of fisheries world-wide (Wilson, 2002). Given the dependence of 1 billion people on seafood as their main source of protein, contamination and degradation of the marine environment will have adverse consequences for health and well-being.

The scale of the challenge facing us was acknowledged in the Oristano Declaration "… *the global coastal environment is under threat through intensified natural resource utilisation brought about by higher densities of settlements, increased shipping, rapidly growing aquaculture production, expanding tourism activities, massive resource exploitation and other activities, nowhere more so than in the coastal marine environment*." All of these issues contribute individually, but more important, cumulatively to higher risks for the global burden of disease (Bowen and Depledge, in press).

THE CHANGING CHARACTERISTICS OF THE MEDITERRANEAN SEA REGION

The population of the Mediterranean countries was about 450 million in 1996 but will increase by ca.100 million by 2030 (EEA, 1999). In 2000, the 22 countries bordering the Mediterranean Sea accounted for 7% of the World's population, 13% of the world GDP (though decreasing as the Asian economies grow), 60% of the worlds water poor people and contributed 8.3% of the CO_2 emissions (Plan Bleu, 2006).The rising population is enhanced annually by tourism, particularly on the shores of the north-western basin. There are approximately 218 million visitors each year who constitute 32% of global tourism. This number is expected to rise to 235–300 million per year in the next 20 years (EEA, 2006). These demographic changes and the way in which people live probably represent the greatest threats to the sustainability of the Mediterranean marine ecosystem. Figure 1 vividly demonstrates the growth in human populations living in coastal towns and cities between 1950 and 1995. From 601 coastal cities with a population of more than 10,000 inhabitants, only 69 % operate wastewater treatment plants. The detoxification and removal of pollutants is often inadequate. Untreated or partly treated urban effluents contain significant loads of nutrients and suspended matter that are contaminated with metals and other pollutants. Especially significant impacts occur in southern and eastern Mediterranean countries in contrast to northern countries where efforts devoted to overcoming the problems generated by the use of chemicals are at least partially successful (EEA, 2006; Plan Bleu, 2006). However, throughout the region there appears to be a lack of political willingness to enforce environmental regulation. The southern Mediterranean in particular is growing at the expense of the natural environment since neither the economic conditions nor the required technologies to support management actions are available. According to the European Environment Agency, the top priority in environmental management in the Mediterranean region is to develop the necessary environmental legislation and to enforce it (EEA, 2006).

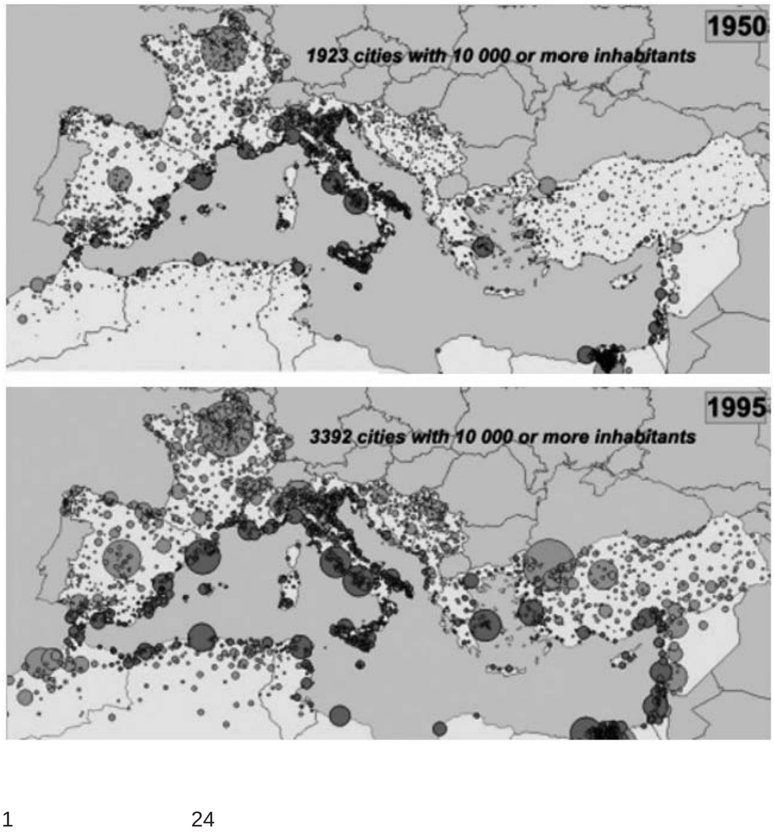

Fig. 1. Changes in the human population distribution in the Mediterranean region between 1950 and 1995 (modified from Plan Bleu, 2006).

Data on the total size of the Mediterranean catchment area are not available. The Nile, Rhône, Ebro and the Po rivers all flow into the Mediterranean Sea. There is also a net inflow from the Black Sea. Overall, however, evaporation exceeds precipitation and freshwater inputs, giving rise to a high salinity in the Mediterranean Sea (CIESM, 2002b; EEA, 2006). The region's seawater is also characterized by having a relatively high temperature (12 to 25 °C), which some have suggested induces high metabolic rates in marine biota (EEA, 2006). In fact, metabolic adjustments probably ameliorate this effect to some extent, but there is no doubt that many marine organisms live close to their thermal limits and oxygen availability is reduced compared to that at lower seawater temperatures. Global warming will exacerbate these effects. The tidal range is less than 50 cm and thus contributes little to mixing, dilution and dispersion of dissolved and particulate polluting wastes. Mediterranean waters are poor in nutrients, exhibit low primary production and low phytoplankton biomass. The fauna and flora is, however, one of the most biodiverse in the world. High numbers of alien species are invading the region, especially via the Suez Canal (EEA, 2006; Plan Bleu, 2006). It is interesting to consider whether these invaders should also be protected from pollutant impacts. If so, then actions should be taken to assess their vulnerability.

One further point that is often overlooked is the impact of dams on the Mediterranean Sea (see CIESM, 2006). Clearly, modification of river flow rates in the region will alter freshwater inputs, nutrient and sediment loads as well as pollutant inputs (see CIESM, 2006). The construction of the Aswan Dam in 1965, for example, resulted in severe reductions in nutrient inflow into the eastern Mediterranean with severe adverse consequences for biodiversity (Azov, 1991). Currently, hundreds of new dams are being planned. In Turkey alone, by 1991 164 large dams and 765 small dams had been completed and put into service to ensure the water supply, irrigation, hydropower and flood control. Environmental modification, such as construction of dams, can change the temperature and humidity of the soil and vegetation, which may result in changes in the spread of infectious parasitic diseases such as leishmaniasis. Notably, the outbreak of cutaneous leishmaniasis in the central and southern parts of Tunisia in 1982-83 occurred following the construction of the Sidi Saad Dam (Ben Rachid and Ben Ismail, 1987).

PAST AND PRESENT STATUS OF MEDITERRANEAN POLLUTION

Land-based sources of Mediterranean pollution include; sewage and urban run-off, urban solid wastes, persistent organic pollutants (POPs), heavy metals, organohalogen compounds, radioactive substances, nutrients, suspended solids and hazardous wastes. Off shore and marine-based pollution is due primarily to petroleum hydrocarbons from shipping activities and marine litter. 30% of international maritime freight traffic and 20-25% of oil maritime transport is via the Mediterranean Sea. Current environmental problems of the coastal regions are summarized in Table 1.

Table 1. Major environmental problems in the coastal zone of the Mediterranean countries.

	Urban effluents	**Urban solid wastes**	**Industrial effluents**	**Oily effluents**	**Stockpiles of toxic chemicals**	**Coastal eutrophication**	**Coastal urbanisation**
Albania	+	+	–	–	+	+/–	+/–
Algeria	+	+	+	+	–	+/–	+
Bosnia and Herzegovina	+	+	–	–	+/–	–	+
Croatia	+	+	–	+ (expected)	–	+	+
Cyprus	+/–	–	+	–	–	–	+/–
Egypt	+	+	+	+/–	–	+	+
Greece	+	+	+	–	–	+/–	+/–
France	+	–	+	–	–	+/–	+
Israel	+	–	+	+/–	–	+/–	+/–
Italy	+	–	+	+	–	+	+
Lebanon	+	+	+/–	–	–	–	+
Libya	+	+	+	+/–	–	–	–
Malta	+	+/–	+/–	+/–	–	–	+
Monaco	–	–	–	–	–	–	+
Morocco	+	+	+	+	+/–	+/–	+
Gaza Strip	+	+	+	–	–	+/–	+
Spain	+	–	+	–	–	+/–	+
Slovenia	+	–	+	–	–	+/–	+
Syria	+	+	+	+	–	+/–	+/–
Turkey	+	+	+	+/–	–	+	+
Tunisia	+	+	+	–	–	+/–	+

+ : Important problem; +/– : Medium problem; – : Small problem

From EEA (2006).

Exposure to, and effects of, endocrine disruptors, heavy metals and persistent organic pollutants are being documented increasingly for organisms such as marine mammals, living in open waters. (Fossi *et al*., 2000; Fossi and Marsili, 2001). Along the Mediterranean coastline, 131 ‘pollution hot spots’ have been identified (UNEP/WHO, 2003). These hot spots are point pollution sources or polluted coastal areas which may affect human health, ecosystems, biodiversity, sustainability, or economy. Of these, 26 % are urban, 18 % industrial and 56 % mixed (urban and industrial) (UNEP/WHO, 2003). Additionally, 59 marine areas under threat of becoming pollution hot spots have also been identified along the Mediterranean coastline (EEA, 2006). An unexpected major threat is posed by stockpiles of hazardous chemicals. For example, lindane stockpiles are located at Durres, Albania. Releases have heavily contaminated the area and rainwater has carried the contaminants into the sea. High concentrations of PCBs and pesticides have since been found in marine samples collected from nearby Porto Romano Bay. The human health impact is imminent as thousands of people recently arrived from other parts of Albania and are now living in and around the highly contaminated area (EEA, 2006).

It is noteworthy that the European Union has defined ‘hazardous substances’ as substances or groups of substances that are toxic, persistent and liable to bio-accumulate; and other substances or groups of substances which give rise to an equivalent level of concern (EU Water Framework Directive 2000/60/EC, Art 2). This definition is perhaps unwise as an enormous number of hazardous substances are not persistent or bioaccumulative, but still leave powerful toxicological legacies following exposure (for example, benzene). Substances tend to persist and bioaccumulate precisely because they do not easily interact with metabolic and physiological processes. Instead, they remain in sanctuary sites, especially fat deposits. This issue clearly needs to be reconsidered.

FUTURE THREATS TO THE MEDITERRANEAN REGION

As well as continued problems with current pollutants, widely recognized, emerging marine issues for the Mediterranean region include biological invasions, overexploitation of fisheries resources, expansion of aquaculture, natural hazards and ecological quality status, and the increasing appearance of Harmful Algal Blooms (HABs), EEA (2006). With regard to the latter, it has been estimated that the socio-economic impact of HABs in Italy, Greece and France is already around 329 million euro per year (EEA, 2006).

The forementioned list presents a rather limited view of future challenges. In the broadest sense, population growth and redistribution, and socio-economic developments, together with climate change are likely to be the greatest threats. More specifically, several new chemicals are beginning to pose problems including organobromine compounds used as flame retardants; for example, the brominated diphenyl ethers used in electronic components, plastics and textiles (Dewailly and Knap, 2006). Halogenated phenolic compounds (HPCs) are also a cause for concern although they are not routinely measured. HPCs include the hydroxyl metabolites of PCBs and pentachlorophenol (PCP). Their distribution in the Mediterranean marine environment is not well known, nor is the extent of the threat they pose to human health. However, in Canada, HPCs have been found in seafood and in the human consumers of the seafood. Dewailly and Knap (2006) also identify perfluoroctane sulfonate (PFOS) as a threat. It is very persistent and appears to be an increasingly common contaminant found in Mediterranean marine mammals (Kannan *et al*., 2005). It is suspected of being an endocrine disruptor but the evidence is inconclusive.

The increase in coastal population densities and the ageing of the northern European population is resulting in the extensive use and release of pharmaceutical agents (Jones *et al*., 2004). This deserves special attention (see Budzinski and Togola, this volume) and may represent a worrying insidious threat to both biota and human beings. For example, the use of cancer chemotherapy agents is increasing by 10% year after year in some countries in northern Europe and residues are now measurable in some European rivers. These will eventually pass to the sea. En route, however, river water is often abstracted for use as drinking water for humans.

As new analytical methods become available with increased sensitivity, there will, no doubt, be many more organic chemicals that raise concerns. However, perhaps more attention should be given to assessment of the biological effects associated with exposure to these chemicals and to complex mixtures of contaminants. Measuring chemical residues, such as the persistent organic pollutants as recommended by the Stockholm Convention, may be far too complex and expensive for many of the countries surrounding the Mediterranean Sea. It seems unwise to add one chemical after another to the list of substances that should be measured.

Biomonitoring studies have been conducted around the Mediterranean Sea for more than 30 years. They involve measuring chemical residues is tissues of marine organisms, usually mussels, thereby demonstrating bioavailability (the Mussel Watch programme, Goldberg, 1975; CIESM, 2002a). These programmes are valuable in that they identify mussels and other seafood in the local area that are unfit for human consumption and they also provide useful information concerning trends in contaminant release and bioavailability. However, they cannot assess the integrated impact of contaminants and environmental stressors on the health of populations and communities of marine organisms, or effects on ecosystem structure and function. For this, the biomarker approach offers a practical, ecologically relevant way forward (Depledge and Galloway, 2005). Biomarkers are biochemical, cellular, physiological or behavioural responses of organisms that signal exposure to or adverse effects of anthropogenic chemicals and radiation (Depledge, 1994). Biomarker response can provide early warning of pollution exposure and effects that then inform management actions. It is essential that suites of biomarkers are used in a weight of evidence approach, and in combination with chemical residue information (Depledge, 1989; Depledge *et al*., 1992).

At least two trends are emerging in biomarker research. The first reflects the realization that both the costs and the availability of expertise may limit the feasibility of measuring either chemical residues or biological responses in organisms in many of the poorer countries around the Mediterranean Sea (and indeed, in developing countries world-wide). This has been addressed through the development of the RAMP programme (Rapid Assessment of Marine Pollution) which is a pilot programme of the Global Oceans Observing System (Depledge, 2000; Galloway *et al*., 2002). It involves the use of rapid, easy to use, inexpensive measurements of chemical pollutants and biomarkers that permit a preliminary assessment of the state of the coastal marine environment.

A second trend to emerge is the development of increasingly sophisticated methods for measuring patterns of biomarker responses. This involves use of the very latest techniques emerging in the fields of genomics and proteomics, and is at present an expensive option. However, it is highly likely that these techniques will rapidly become cheaper, simple to use and robust (see papers by Hamilton and Bebbiano, this volume).

The threat posed by plastics in the Mediterranean Sea is growing. Millions of metric tons are produced annually and significant quantities end up in the marine environment (Galgani *et al*., 2000). In some parts of the Mediterranean Sea enormous numbers of plastic bags have accumulated offshore resulting in the death of marine mammals, fish and invertebrates. More worrying still is the finding that as plastic wastes degrade they are often reduced to fine and ultrafine particles that become incorporated in sediments (Thompson *et al*., 2004). These particles in turn are ingested by deposit feeders. Effects are largely unknown but early evidence suggests that plastic particles do bind chemical pollutants to their surfaces and may affect bioavailability.

Trends in chemical production in the future warrant closer inspection. Many chemical manufacturers envisage that their activities will be geared more to the needs of an estimated 1 billion new consumers in Asia over the next 10-20 years. In Europe, a different pattern is starting to emerge with successful advances in nanotechnology and in producing genetically modified organisms (Royal Society, 2002, 2004; Owen and Depledge, 2005). Up to 25% of industrial manufacture will be achieved using nanotechnologies with investment in excess of US$16 trillion anticipated over the next 10-20 years. New antifouling materials involving nanoparticle coatings are already being tested on boats, booms and other structures in the marine environment. Cerium oxide nanoparticles added to diesel fuel increase fuel efficiency by up to 30% in land-based vehicles and plans are underway to use them in shipping. There are many other examples of nanotechnology applications, but as yet little attention has been paid to the likely fate and effects of nanomaterials in the marine environment.

The political situation in several countries surrounding the Mediterranean Sea is unstable and is a cause of concern, not only in its own right, but because of potential pollution impacts. Oil spills resulting from the recent war in Lebanon provide one such example.

POLICY CHALLENGES

The key challenge in developing policies to combat pollution threats in the Mediterranean Sea and elsewhere is to ensure that an integrated approach is adopted based on the best available scientific evidence. Assessing the toxicity of individual chemicals, setting safe limits and monitoring their concentrations in the environment will not suffice. Exposure to pollutants invariably occurs in complex mixtures, for example following the discharge of sewage or industrial effluents. In the coming years the bioavailability of pollutants will be influenced by rising seawater temperatures and increasing acidification. It is easy to imagine how this might increase the bioavailability of some heavy metals, but there may also be effects on the bioavailability and persistence of organic pollutants.

The changing nature of pollution threats in the Mediterranean Sea will have to be considered and factored into new policies and regulations. Climate change will affect precipitation rates and outflow from catchments discharging into Mediterranean coastal areas. This in turn, will influence both the timing of release and the amounts of pollutants delivered via this route. The types of pollutants discharged will also change significantly. Policies and regulations will have to address the socio-economic changes that occur as industrial manufacture over the coming years shifts increasingly from northern Mediterranean countries to those in the south, or more likely still, to other regions of the World, especially Asia. A range of complex mechanisms link chemical pollution to human health and well-being and these are of growing significance in affluent Mediterranean countries. Impacts of chemicals on human health not only occur via the food supply or air pollution, but also indirectly through damage to coastal ecosystems. In the southern Mediterranean, the METAP (Mediterranean Environmental and Technical Assistance Program) has provided valuable insights in the relationships between environmental pollution, ecosystem degradation, economic burdens and threats to human health and well-being (METAP, 2006). Algerian coastal communities east of the capital Algiers provide examples of both heavily degraded coastal areas and coastal areas that have been relatively well preserved. Studies show that uncontrolled urbanization and poorly regulated environmental pollution are the main causes of degradation costs. Urbanization and industrial installations create economic deficits by polluting water and air and by generating waste. Total costs were estimated to be between 4 and 9 % of the GDP of the pilot area. The greatest costs were related to effects of coastal degradation on health. This included losses of recreational opportunities, ecosystem services, in particular availability of ground water, and productivity of agricultural and natural systems (METAP, 2006).

There is growing evidence that humans benefit from spending time in natural ecosystems (see Depledge, in press). Exercising and relaxing in natural environments have important health benefits and have been shown to be valuable in the treatment of mental disorders. The financial consequences can be quantified and are significant.

All of the above need to be reflected in a new suite of integrated environmental, human health and long term economic policies for the Mediterranean region. If natural and aesthetically pleasing ecosystems continue to be lost as a result of pollution and habitat destruction then Mediterranean countries and their populations will be the poorer.

Dedication

This paper is dedicated to the memory of my mother, Edna May Depledge (1926-2006).

Pharmaceutical substances: emergent contaminants in marine and estuarine systems

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INTRODUCTION

Beside the principal classical chemical contaminants (PAHs, PCBs, pesticides, phtalates, trace metals, dioxins, etc.), we find in aquatic environments substances such as pharmaceutical substances (Ternes *et al*., 1998; Hirsch *et al*., 1999; Ternes, 2001; Kolpin *et al*., 2004; CIESM, 2004; Tauxe-Wuersch *et al*., 2005). Beyond them we can consider various classes according to their therapeutic action: hormones, antidepressants, analgesics, antibiotics, lipid regulators, etc. Important quantities of these molecules are consumed in our western societies (Table 1) and are rejected *in fine* in the aquatic media via sewage treatment plants (incomplete destruction) (Joss *et al*., 2005; Castiglioni *et al*., 2006). They are increasingly studied as they could represent a non negligible environmental risk when considering on one hand the potentially important quantities introduced in the aquatic media and on the other hand the fact that they have been designed in order to be biologically active. These compounds could have important toxic effects (Sausen *et al*., 1995; Brooks *et al*., 2003; Schwaiger *et al*., 2004; Mimeault *et al*., 2005) towards aquatic organisms but in order to estimate environmental risks there is a need for data documenting the effective contamination of the aquatic environment by these molecules (Webb, 2001; Khan and Ongerth, 2004; Schwabb *et al*., 2005).

Table 1. Consumed quantities expressed as tons per year (NSAID: Non Steroidal Anti-Inflammatory Drug).

Compounds	Therapeutic class	UK (2000) (a)	Germany (1995-1997) (b)	Australia (c)	France (d)
Paracetamol	Analgesic	2000	-	295	2294
Aspirin	NSAID	770	> 500	20	880
Ibuprofen	NSAID	-	105-180	14	166
Erythromycine	Antibiotic	27	-	11	
Ketoprofen	NSAID	-	0,7	4	
Diclofenac	NSAID	26	75	4	39
Penicilline V	Antibiotic	22	140	9	

(a) (Webb, 2001).
(b) (Hirsch *et al.*, 1999; Ternes, 2001; Ternes *et al.*, 1998).
(c) (Khan and Ongerth, 2004).
(d) (Janex *et al.*, 2002).

The present work concerns analytical developments in order to analyze different classes of pharmaceuticals in aquatic media (dissolved phase, particulate matter, biological tissues). These developments involve both extraction and purification methods such as SPE and microwave assisted extraction but also analytical developments for identification and quantification by GC/MS. The work presented deals with the development of an extraction procedure that makes it possible to measure at trace level (ng.l^{-1}) several pharmaceuticals belonging to very different chemical classes: anti-inflammatory drugs, antidepressants, hypolipidic drugs, etc. Reliability and sensitivity have been tested on 18 different compounds (7 neutral compounds and 11 acidic drugs) extracted simultaneously and analyzed with two GC-MS methods. Different applications demonstrate the multi-residue but also multi-matrix characteristics of the developed method.

All the analytical developments have been applied to several environmental case studies. Various French estuaries (Seine, Loire, Gironde, Adour) have been studied as well as marine locations (Marseille coast). In all cases it has been possible to detect quite important concentrations of pharmaceutical substances.

The use of semi-permeable membrane devices (POCIS type) in order to get access to integrative sampling procedure (necessary when considering the variability of aquatic contamination) has been also investigated. The aim of the study was to determine the sampling rates (Rs; expressed as effective volumes of water extracted daily) of POCIS device for 14 pharmaceuticals in several conditions of temperature, salinity and analyte concentration. Following laboratory experiments, environmental application has been managed, showing the application of POCIS devices in the case of a contaminated system: the Seine Estuary.

MATERIAL AND METHODS

Raw water was filtered on GFF filters to separate dissolved phase and particles. For natural waters, one l was filtered; for Wastewater Treatment Plant Effluent (WWTP effluent), 500 ml were used for each extraction. pH adjustment was made just before extraction processes at pH 2 with HCl (3.5 M) and internal standards (diazepam d^5, amitryptiline d^6, nordiazepam d^5; 25 µl to 50 µl of a methanolic mixture containing 1 µg.g^{-1} of each standard) were added to the samples.

SPE cartridges were conditioned subsequently with 3 ml of ethyl acetate and 3 ml of milliQ-water at adjusted pH 2. Water was percolated under vacuum at a flow rate of 12 to 15 ml.min^{-1}. Cartridges were dried under vacuum for 1 h. After elution with three successive solvents, respectively 3 ml of ethyl acetate, 3 ml of ethyl acetate/acetone (50/50; v/v) and 3 ml of ethyl acetate/acetone/ammonium hydroxide (48/48/2; v/v/v), the samples were completely evaporated under nitrogen and transferred into GC injection vials in 50 to 100 µl of ethyl acetate. For recovery control, pyrene was added to the final extracts for neutral compounds before GC-MS analysis and 1-hydroxypyrene was added to the final extracts for acidic compounds before the derivatization step, consisting in adding 30 µl of MSTFA before incubation at 65 ºC.

For particulate phase or sediment, samples were first freeze-dried until entire dryness, spiked with internal standards and then extracted by focused microwave assisted extraction (Microdigest 301, Prolabo, Fontenay-sous-Bois, France): 30 W, 10 min., in 30 ml of acetonitrile/pH 2 deionized water (70/30; v/v). The extracts were filtered on glass fibre cotton and solvent was evaporated under evaporation assisted by heating and under vacuum (RapidVap, Prolabo, Fontenay-sous-Bois, France) at 80 °C, 500 mbars for 40 min. Aqueous residues were then dissolved in 30 ml of pH 2 deionized water and treated as aqueous sample, as dissolved phase.

RESULTS AND DISCUSSION

Analytical developments

The different kinds of studied water present various organic matter contents and matrix complexity. The limits of detection are very different depending on the origin and kind of water. They vary between 0.1 and 1.5 ng.l^{-1} for tap water, between 0.1 and 2.5 ng.l^{-1} for surface water and between 3.2 and 28 ng.l^{-1} for wastewater, with variability depending on compounds, presented in Table 2. These low detection limits have allowed to quantify pharmaceuticals in various natural environments.

Table 2. Limits of detection obtained for natural waters (expressed in ng.l^{-1}, for one l extracted for tap and surface waters, 500 ml for wastewater effluent).

	Tap water	Surface water	Waste water effluent
Amitryptiline (ami)	0.7	2.2	3.4
Aspirin (asp)	0.2	2.1	7.8
Caffeine (caf)	1.5	2.5	14.3
Carbamazepine (cbz)	0.8	1.4	11.6
Clenbuterol (clen)	0.6	0.3	2.0
Diazepam (dzp)	0.4	1.4	6.9
Nordiazepam (ndzp)	0.4	1.4	6.9
Diclofenac (diclo)	0.9	0.7	4.5
Doxepine (dox)	0.7	2.1	8.3
Gemfibrozil (gemf)	0.1	0.3	1.6
Ibuprofen (ibu)	0.1	0.1	2.4
Imipramine (imip)	0.7	1.2	6.9
Ketoprofen (keto)	0.3	0.7	5.8
Naproxen (nap)	0.1	1.0	3.1

Complete pharmaceutical extraction has been evaluated on different spiked samples: dissolved phase and particulate matter. Figure 1 shows yields of extraction obtained for these two kinds of matrices. For the main compounds, results obtained on solid matrices are comparable with those obtained for aqueous samples. For some compounds, step multiplication decreases yields of extraction (for aspirin, imipramine, terbutaline and salbutamol). The detection limits for the solid phase have been evaluated between 0.2 ng.g^{-1} and 20 ng.g^{-1}.

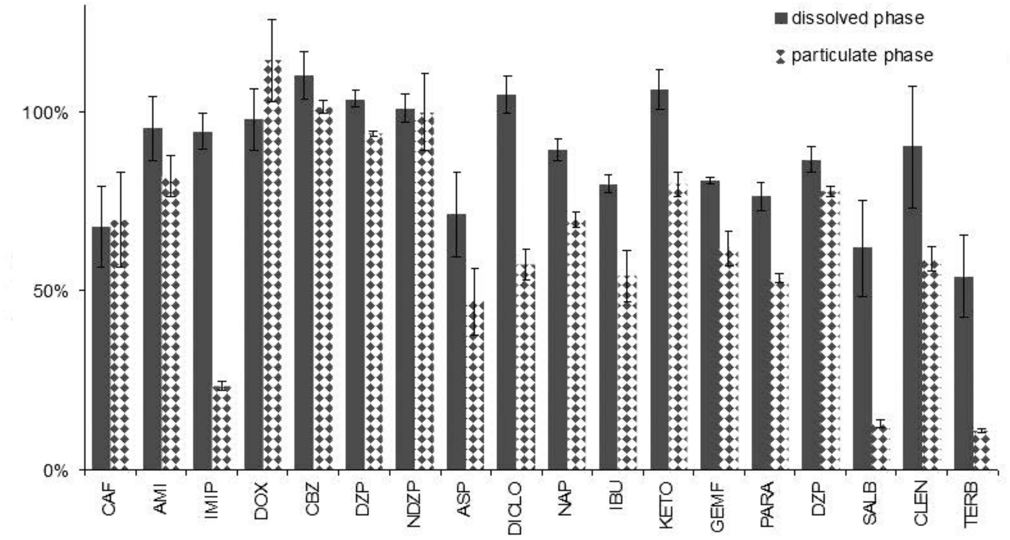

Fig. 1. Yields of whole extraction for two kinds of matrices: dissolved phase and particles (n=3).

Application to Atlantic French estuaries

Measured concentrations fluctuate between a few nanograms per liter and hundreds of nanogram per liter depending on compounds, sampling stations and seasons (Table 3). The Gironde Estuary is the less impacted. Despite the fact there are two big cities (Bordeaux and Toulouse) located in the river basin, the important flow limits the impact of STP. It is the same situation for the Loire Estuary where the influence of the STPs is only noticed close to the STP effluents. The Adour is a small estuary with cities counting variable quantities of inhabitants. The concentrations are quite low. The Seine Estuary appears the most impacted in relation with its high human density and high industrial activity.

Table 3. Concentrations measured for the different estuaries.

	Gironde	**Loire**	**Adour**	**Seine**
Aspirin	< 2 - 4	< 2 - 27	8 - 28	< 2 - 196
Caffein	< 1 - 31	< 1 - 73	< 1 - 16	40 - 860
Diclofenac	< 2 - 4	< 2 - 6	8 - 23	8 - 380
Gemfibrozil	< 2 - 4	< 2 - 5	< 2 - 9	3 - 126
Ibuprofen	< 2 - 3	< 2 - 9	14 - 37	5 - 610
Ketoprofen	< 2 - 24	< 2 - 9	< 2 - 3	3 - 78
Naproxen	< 2 - 7	< 2 - 8	1 - 6	3 - 185
Carbamazepine	< 1 - 13	< 1 - 228	< 1 - 8	3 - 164

When considering this estuary, the main inputs come from Paris in the upstream (Figure 2) especially for the most STP degradable compounds. For the less degradable (diclofenac, carbamazepine, ketoprofen) the inputs from the local STP are more important and account for half of the total inputs.

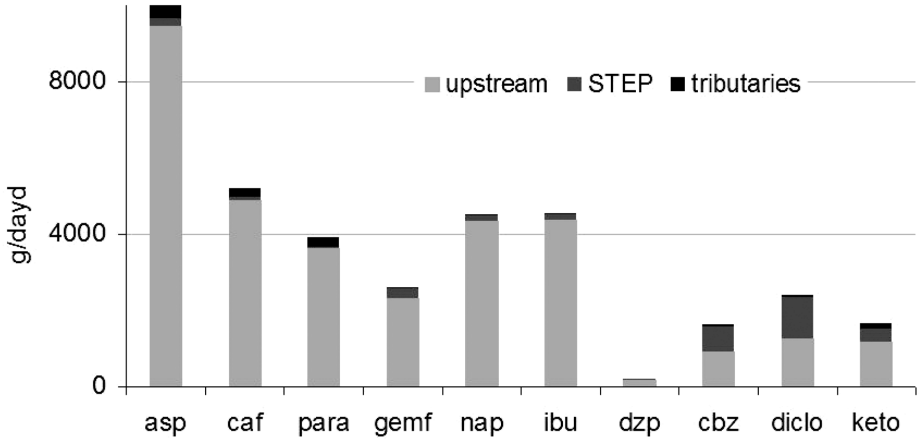

Fig. 2. Relative importance of the various inputs (tributaries, upstream, STP) for the Seine Estuary.

The results have shown that, if the dissolved phase is the most contaminated, the particulate phase could have a large part in the pharmaceuticals spread in aquatic systems (Figure 3). When pharmaceuticals occurrence in solid phase is observed, expressed in ng.g$^{-1,}$ some phenomena can be highlighted. High contents have been measured in the upper part of the Seine Estuary system (dam of Poses), with concentrations up to 1,220 ng.g^{-1} for ketoprofen or 260 ng.g^{-1} for naproxen. The solid phase can participate at a quite important extent to the water column contamination.

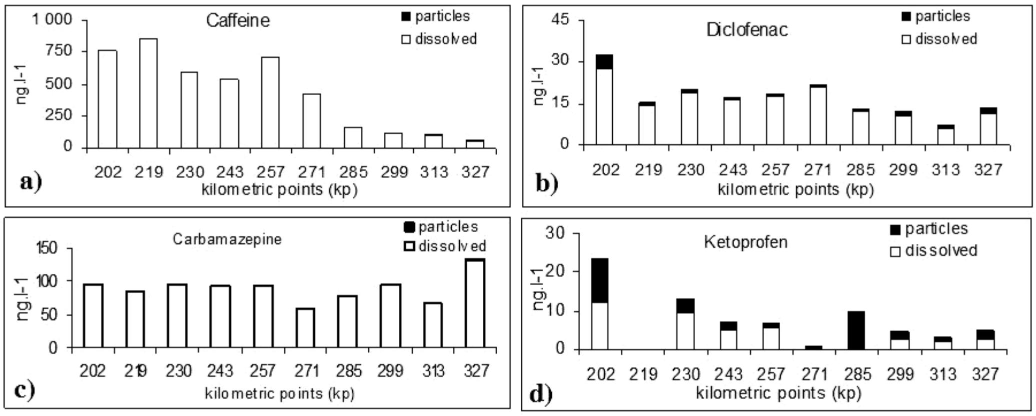

Fig. 3. Pharmaceutical partition between dissolved and particulate phases.
a) caffeine; b) diclofenac; c) carbamazepine and d) ketoprofen.

Case of the Mediterranean Sea: application to Marseilles area (French south coast)

Seawaters, highly contaminated by the Marseilles wastewater treatment plant effluent and with a high organic matter content, have been monitored in the Cortiou rocky inlet, in the Mediterranean Sea (Marseilles area, South coast of France).

Concerning the Cortiou rocky inlet (Figure 4), the measured values are interesting for several reasons. Marseilles wastewater treatment plant has an important capacity (85,500,000 m^3 treated and 1,300,000 population equivalents), without secondary treatment. In France, it is the most important station of this kind, allowing evaluation of WWTP effluent in the worst conditions. This station should be renovated in 2007; the results obtained here allow future comparison studies and monitoring of remediation processes.

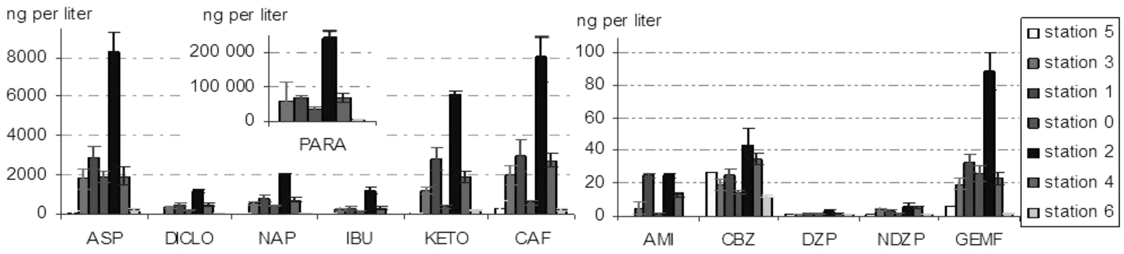

Fig. 4. Pharmaceutical concentration measured in the Cortiou rocky inlet.

Some compounds, rarely found in surface water as well as in wastewater effluents, have been measured around 10 $ng.l^{-1}$, especially for the antidepressants amitryptiline, diazepam and nordiazepam. The high concentration level of treated wastewaters is explained by the relationship between pharmaceutical consumption and occurrence in effluent. Considering 1,300,000 inhabitants, the number of antidepressant consumers is high, increasing the content of those drugs in effluent and then allowing their detection. For the same reasons, very high amounts of non-prescribed drugs have been measured, such as aspirin (8 $\mu g.l^{-1}$), caffeine (8 $\mu g.l^{-1}$), and above all paracetamol (200 $\mu g.l^{-1}$). The degradation of these three compounds is highly related to biological treatment efficiency in wastewater treatment plants (Pedersen *et al*., 2005; Joss *et al*., 2005). In the absence of biological treatment in the Marseilles WWTP, high concentrations of those compounds are thus measured in the effluent.

Concerning the plume of dilution of the effluent and its impact on the Cortiou rocky inlet, this preliminary study focused on the area close to the emissary, more or less 500 meters from the emissary for the remotest stations (stations 5 and 6). Currents influence the plume of dilution and carry effluents towards one side more than towards the other. This very important effluent, introduced into a semi-open aquatic system, poorly submitted to dilution phenomena can present a high environmental risk for aquatic organisms living in the area. Considering the first results documenting the occurrence of antidepressants in fish living in an effluent-dominated stream (Brooks *et al*., 2005), studies focusing on fish exposure and toxicological impact related to this exposure need to be undertaken.

Integrative samplers

Integrative passive sampling is a new approach developed for environmental monitoring. An alternative way to achieve a time weighted average concentration is to use passive samplers. The use of Polar Organic Chemical Integrative Sampling (POCIS) has been recently documented for the detection of pharmaceuticals in the environment (Alvarez *et al*., 2004; Jones-Lepp *et al*., 2004; Petty *et al*., 2004). There is a need for laboratory data to extend the use of this type of tool to new compounds. The aim of the present work was to determine the sampling rates (Rs; expressed as effective volumes of water extracted daily) of POCIS devices for 14 pharmaceuticals in several conditions of temperature, salinity and analyte concentration. These values are influenced by significant changes in water temperature, salinity. Overall POCIS Rs values are independent from aqueous concentrations. After laboratory experiments, an environmental field study has been performed, implementing POCIS devices in the Seine Estuary

(North Atlantic coast of France) and testing the qualitative and quantitative application of POCIS devices on the contaminated system. Figure 5 shows analyte concentration (in ng.g^{-1}of sorbent) obtained after three days of exposure at the dam of Poses in the Seine Estuary. These results show good reproducibility for analyte accumulation, with variability below 20 % between the three devices despite variable environmental conditions.

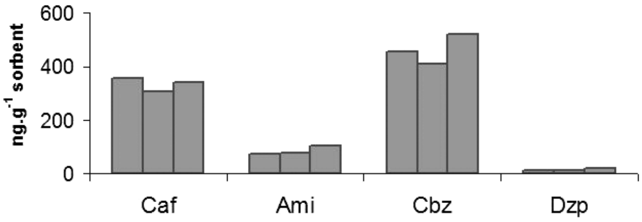

Fig. 5. Accumulation for three days of exposure in the case of the Seine Estuary experiment.

The use of sampling rates obtained in laboratory has led to calculate the concentrations for four analytes that are shown in Figure 6. They are compared to averages of measured concentrations during the same time by classical methodology (3 days, one sample per day). During a short time of exposure, POCIS gives relative good quantifications for pharmaceuticals: for carbamazepine, the measured concentrations are 35 ± 3 ng.l-1 versus 41 ± 5 ng.l-1 for calculated concentration; for caffeine and diazepam, similar results have been obtained, with a larger difference for diazepam due to quantification near the detection limits for the classical methodology. For amitryptiline, the measured values are under the limits of detection for the classical methodology, while POCIS sorbent analysis allows analyte identification and quantification, showing the accumulative property of POCIS tools. By increasing the quantity of extracted compounds, it should be possible to detect not easily detectable compounds by discrete sampling.

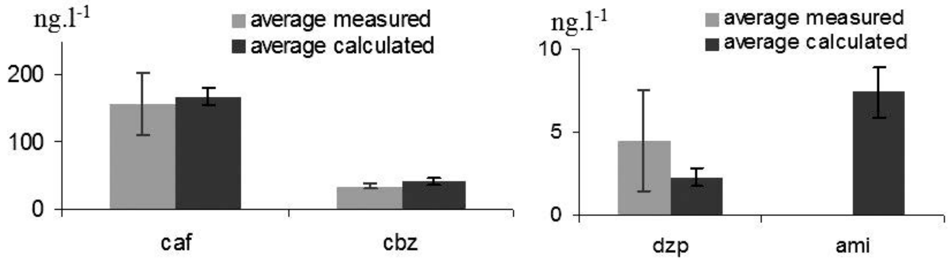

Fig. 6. Comparison of calculated and measured concentrations for three days POCIS exposure in the Seine Estuary (n=3).

The suitability of the devices for monitoring multiple media under a wide range of environmental conditions has been established. The uniformity or reproducibility of the sampling matrix and, on the other hand, the ability to detect compounds at low concentration levels below detection limits of discrete sampling have been highlighted.

CONCLUSION

In all cases it has been possible to detect quite important concentrations of pharmaceutical substances. Measured concentrations fluctuate between few nanograms per liter and hundreds of nanograms per liter depending on compounds, sampling stations and seasons. The results have shown that, while the dissolved phase is the most contaminated one, the particulate phase could have a large part in the pharmaceuticals spread in aquatic systems. The understanding of the transfer of these compounds to aquatic organisms and of their toxicity is under progress, as well as their impact on human health in relation to environmental contamination. There is really very few data at this moment on this aspect and investigations are badly needed in order to gain a better knowledge. When considering antibiotics the bioaccumulation properties of some of them are known in relation with their use in aquaculture and there are, in this case, EU norms fixing their maximum level for products consumed by humans. For the other pharmaceutical classes the current state of knowledge is very low; some studies have just shown the possibility of trophic transfer, illustrating the strong need of additional work (Lai and Scrimshaw, 2002; Schwaiger *et al*., 2004).

Effects of contaminants in marine organisms: from the multibiomarker approach to proteomics

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ABSTRACT

The increase of contaminant levels as complex mixtures in the marine environment is placing the health of marine organisms as well as human health at risk. To asses the effects of these contaminants a multibiomarker approach was applied using selected bioindicator species. A battery of biomarkers of exposure, defence and susceptibility was used to assess the pollution effect to two mollusc species *Mytilus galloprovincialis* and *Ruditapes decussatus* on the Southern coast of Portugal. Although the results were able to differentiate the effects of contaminants in mussels and clams from the different sites, the biomarkers might not be enough to highlight the whole effects of the contaminants in the cells. Therefore, the use of the proteomic approach can provide new insights into the biological responses and on new environmental challenges.

INTRODUCTION

Over the last decades, levels of contaminants in the marine environment have increased as a consequence of human activities, posing a risk to human health. The main sources of marine pollution include: municipal, industrial and agricultural wastes and run-off, sewage effluents, sand and gravel extraction, exploitation of oil, gas and solid minerals, placement of cables and pipelines, tourism, recreational activities, aquaculture, fisheries, maritime transport and accidental spills. Therefore, organic and inorganic contaminants resulting from these activities are present in the marine environment as complex mixtures that, even at very low concentrations, can elicit damaging effects whose assessment is difficult to interpret. Therefore, there is a need to develop new methods for the identification, estimation, comparative assessment and management of the risks posed by these pollutant discharges to the marine environment (Cajaraville *et al.,* 2000; Broeg *et al*., 2005).

Although chemical analyses of these contaminants are necessary to evaluate pollution, assessment of marine pollution cannot solely be based on chemical analysis because this approach does not provide any indication of deleterious effects on the biota (Cajaraville *et al*., 2000; Allan *et al.,* 2006). Therefore, the use of marine organisms was considered the most appropriate matrix for the evaluation of health risk, since animal responses integrate the bioavailable concentration of contaminants in time and space. In addition, the increasing number and types of potential pollutants entering the marine environment and their presence as complex mixtures call for novel strategies to access the health effects of these contaminants. Therefore, to analyze the extent of the disturbances in biological systems and to quantify its health state, the integration of several biological effects at different levels of biological organization is needed (Allen and Moore, 2004; Broeg *et al*., 2005).

The use of biomarkers measured in body fluids, cells or tissues to detect the presence of contaminants (exposure biomarkers) or the magnitude of the host response (effect biomarkers) was considered a promising approach since they respond to either (or both) exposure to and/or doses of xenobiotic substances. In order to categorize biomarkers according to their type of response, De Lafontaine *et al*. (2000) proposed to refer to them as biomarkers of 'defence' (e.g. the induction of MT or EROD response to specific classes of contaminants: the first to metals and the second to coplanar organic chemicals, particularly polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) is indeed a defence reaction by an organism) or as biomarkers of 'damage' (e.g. DNA strand breaks, Acetylcholinesterase (AChE) inhibition, lipid peroxidation (LPO) associated with oxidative perturbations, particularly within the polyunsaturated lipid-rich cell membranes or imposex induction). Non-specific biomarkers (such as catalase (CAT) activity and other antioxidant enzymes) are also important because they can indicate whether if an organism was submitted to a particular environmental stress, for instance, oxidative stress. Among other responses, molecular biomarkers also include the induction of biotransforming systems or the expression of their encoding genes, the levels of damaged biomolecules or the increased activity of their repair mechanisms. On the other hand, responses at higher levels of biological organization are directly indicative of ecosystem health and hence, relevant to environmental management.

Biomarkers are affected by the presence of mixtures of different chemicals in contaminated areas giving rise to additive, synergistic and/or antagonistic effects. In spite of these, several biomarkers were included in environmental pollution assessment by several international organizations and governmental agencies (Galloway *et al.,* 2004; Schiedek *et al.,* 2006). Therefore, the assessment of the biological effects should be based on a battery of different biomarkers, since the use of a single biomarker in environmental studies does not reflect the integrated response of the organism to the environmental stressors (Galloway *et al*., 2004; Broeg *et al.,* 2005).

A battery of selected biomarkers was used to assess the pollution effect to two mollusc species on the Southern coast of Portugal. These include the mussel *Mytilus galloprovincialis,* a common bioindicator model in marine pollution assessment studies, and the clam *Ruditapes decussatus,* a marine mollusc of economic relevance particularly in the Mediterranean region and proposed as a bioindicator species in areas where mussels are not available (Bebianno *et al.,* 2004). Mussels (sites 1-8) and clams (sites 4-6) were sampled in winter from hot spot areas previously identified in the southern Portuguese coast (Bebianno and Machado, 1997) (Figure 1).

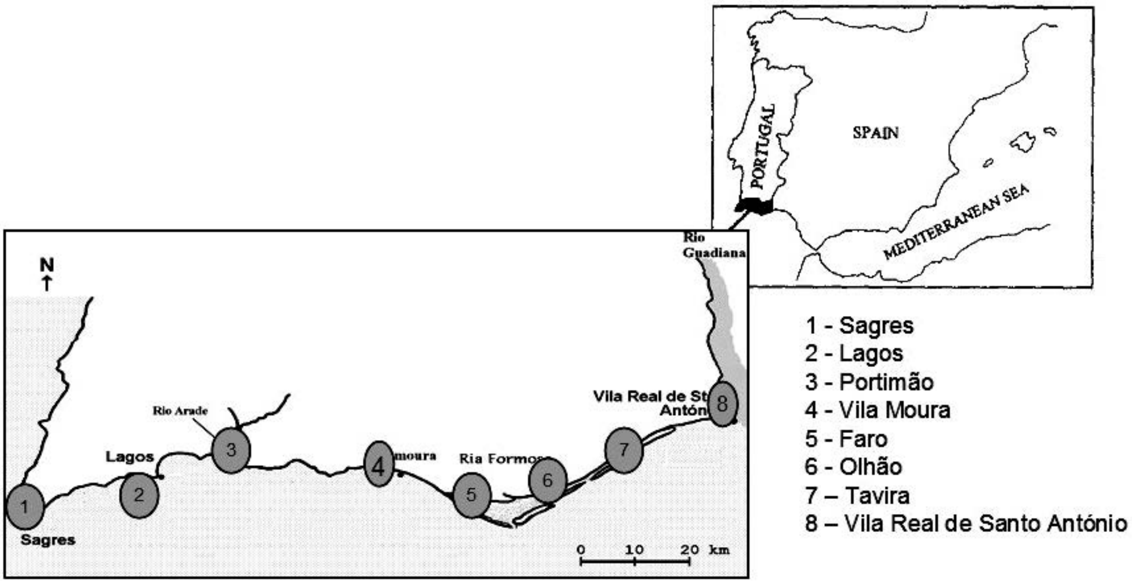

Fig. 1. Location of the sampling sites along the South Coast of Portugal.

Several biomarkers: (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx) (total and selenium-dependent), lipid peroxidation (measured as MDA+4HNE, one of the final products of lipid peroxidation), cytochrome P450 (CYP 450) glutathione S-transferase (GST), ALAD, MT and AChE were measured in different tissues of the mussel *Mytilus galloprovincialis* and the clam *R. decussatus* that comprises a multibiomarker approach to assess environmental changes in the southern coast of Portugal and the impact on the health of these species. Figure 2 presents the levels of these biomarkers in the digestive gland, of the mussel *Mytilus galloprovincialis*. Except for TGPX, all the other biomarkers show variability among sites. PAHs measured in the same organisms were directly related with cytochrome P450 (Figure 3). When the same biomarkers were measured in the clam digestive gland although space variability was also observed, the behaviour of these biomarkers was different from that of the mussels indicating species specificity (data not shown). Moreover using GST-pi gene (Mg GST-p) from gills and digestive gland of *Mytilus galloprovincialis* collected in the same places revealed tissue and space dependence suggesting that GST-pi gene (Mg GST-p) could be a useful indication of the biotransformation of organic contaminants in these tissues (Figure 4).

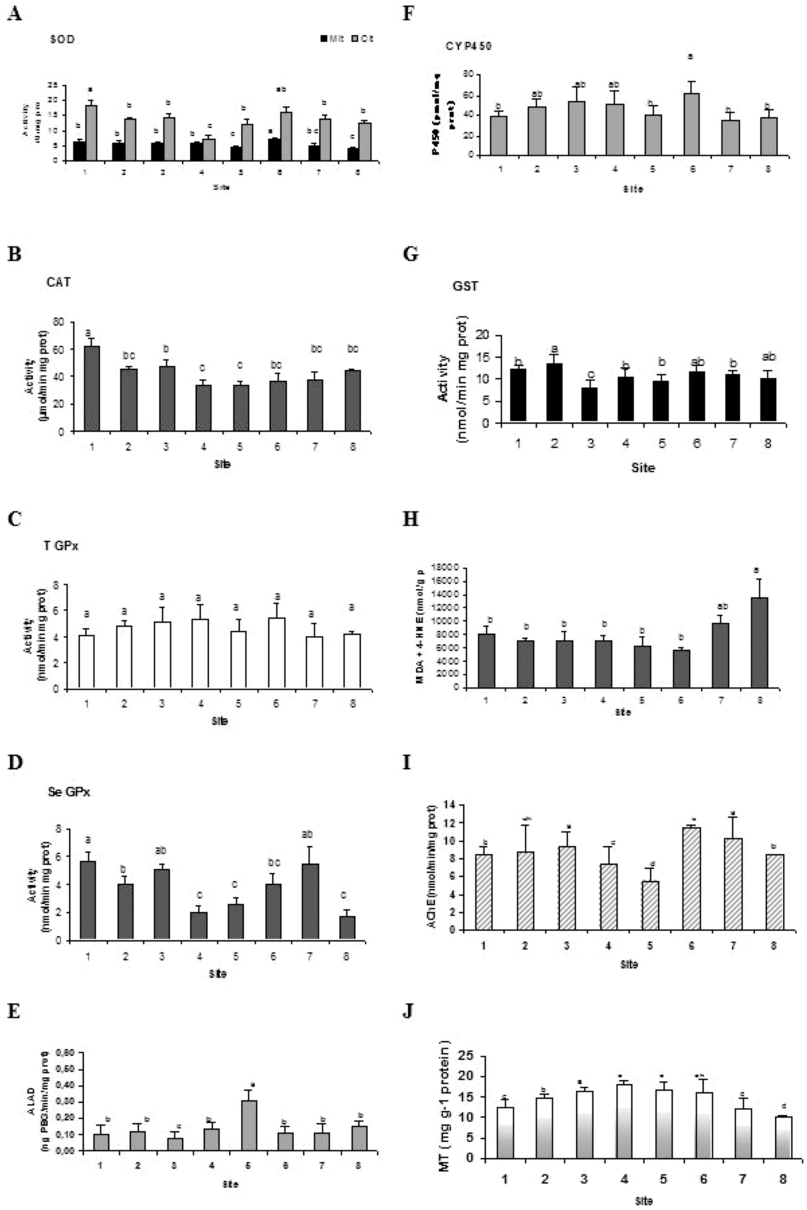

Fig. 2. *Mytilus galloprovincialis.* Levels of Biomarkers: (A) SOD mitochondrial and cytosolic; (B) CAT; (C) TGPX; (D) Se-GPX; (E) CYP 460; (F) GST and (H) AchE in the mussels digestive gland collected along the Southern Portuguese Coast (data from BIOTA project).

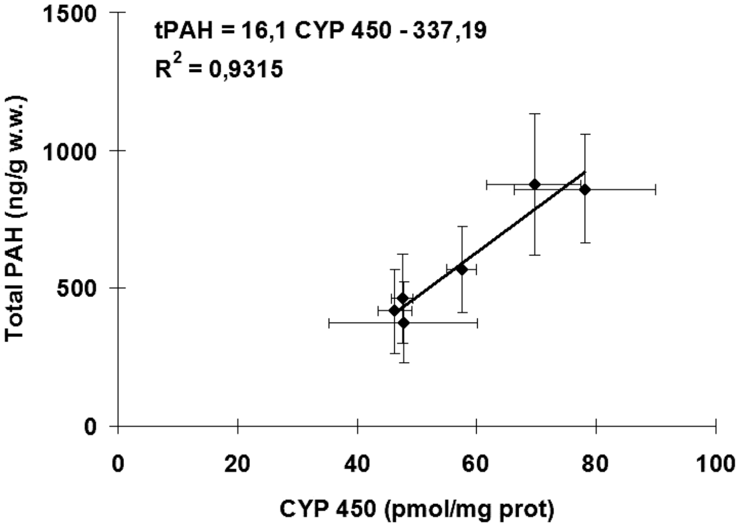

Fig. 3. Relationship between total PAH concentration and cytochrome P450 total content in mussels *M. galloprovincialis,* collected along the south coast of Portugal (adapted from Bebianno *et al.,* in press).

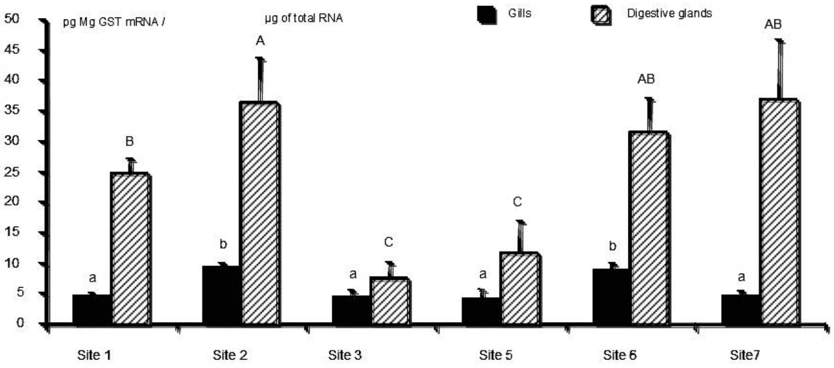

Fig. 4. GST-pi gene (Mg GST-p) expression from gills and digestive gland of *Mytilus galloprovincialis* collected along the southern coast of Portugal. Small letters were used for the gills and capital letters for the digestive gland (adapted from Hoarau *et al.,* 2006).

The results from the different biomarkers were integrated to correctly evaluate the effects of pollution on the health of these aquatic organisms and constitute the first multibiomarker approach study in this area. However, up to now, few studies have used indices to integrate the response of various biomarkers (Viarengo *et al,*. 2000; Narbonne *et al*., 2001; Broeg *et al*., 2005; Dondero *et al*., 2006).

Although the results were able to differentiate between the effects of contaminants in mussels and clams from the different sites, these biomarkers might not be enough to show the whole effects of the contaminants in the cells. The use of conventional biomarkers requires a complete knowledge of the toxic mechanisms of pollutants. They are somewhat biased, because they can only give information about few protein changes, excluding many others also altered by pollutants, but not detected with this approach. Therefore, the use of proteomics (the quantification of all proteins expressed at any individual time in a cell) seems a promising tool. This new technology is able to identify many proteins whose expression is altered by pollutants, without requiring previous knowledge of their toxic mechanisms.

The application of proteomics to environmental studies started when unique protein expression signatures were obtained, after a 2-D gel electrophoresis analysis of mussels *Mytilus edulis* tissues exposed to PCBs and to copper (Shepard and Bradley, 2000; Shepard *et al*., 2000) and of rainbow trout (*Onchorhynchus mykiss*) exposed to endocrine disrupting compounds (nonylphenol, diazonin and propetamphos) detected in sewage effluents (Bradley *et al.,* 2002) and more recently of marine algae *Nannochloropsis oculata* exposed to Cd (10 µM) (Kim *et al*., 2005). Similarly, using 2-D gel electrophoresis, the proteome (protein complement expresses by a genome) of the gills and digestive gland of the clams *Ruditapes decussatus* revealed significant differences between tissues. When clams were exposed to cadmium (40 µg/l) for three weeks, the comparison of the proteome of gills and digestive gland between unexposed and Cd exposed clams revealed that Cd exposure induce changes in more than 500 proteins in the two tissues (Chora, own data) while up to now only metallothioneins and antioxidant enzymes are known to be directly related to cadmium pollution in this species (Bebianno *et al*., 1993; Simes *et al*., 2003; Serafim and Bebianno, in press).

Although the use of proteomics in environmental studies is still very limited, due mainly to the difficulty of identifying proteins from non-model-organisms, this new technology may be a very promising tool to assess the health effects of pollutants on marine species.

Acknowledgements

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Use of toxicogenomics to investigate the effects of toxicants in aquatic systems

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The advent of new genomics tools has provided a powerful new way to examine low level effects of chemicals on biological systems (Bates *et al*., 2005; Hamilton, 2006). It is clear from recent data that changes in gene expression at the genomic level represent one of the most sensitive measures of low level effects currently available, providing an endpoint that can detect responses at doses that are often several orders of magnitude lower than concentrations required to see a response with more traditional toxicological endpoints (Andrew *et al*., 2003; Davey *et al*., in press). However, because of this sensitivity there has been considerable debate as to the toxicological significance of such low level changes in gene expression. In particular, it is not yet clear whether such low dose gene alterations are reflected in concomitant alterations in protein expression or function, cell phenotype or other downstream effects that might be linked to actual biological responses at the physiological or pathophysiological level (Bates *et al*., 2005; Hamilton, 2006). Such concerns have so far precluded the application of toxicogenomics endpoints in the human risk assessment process or as biomarkers of environmental or ecological impact. It will therefore be important to determine the relationship between specific gene expression changes and downstream events, and to determine whether such changes reflect an adaptive response, a normal physiological response, or a pathophysiological response (Shaw *et al*., 2006; Stanton *et al*., 2006).

Nonetheless, toxicogenomics can provide a means of determining characteristic patterns of gene alteration that can be used to assess low level exposures, to identify potential biomarkers of exposure and effects, and in defining a true “no effects” level (Bates *et al*., 2005; Andrew *et al*., 2003; Bodwell *et al*., 2006). Toxicogenomics studies are also useful mechanistically both for hypothesis testing and for hypothesis generation, but must ultimately be linked to other “downstream” endpoints in order to determine their toxicological significance (Stanton *et al*., 2006). It will also be important to develop model organisms that can serve as sentinels for environmentally relevant exposures and their biological consequences. Such models should be ecologically relevant, preferably serve both as sentinel species and as keystone species, be widely distributed, be amenable to study both in the laboratory and in the field, have adequate genomics resources available, and also provide important mechanistic information at the biochemical and physiological levels but also at one or more ecological levels. Two examples of such useful models that have recently been developed are the freshwater zooplankton, *Daphnia pulex* (Shaw *et al*., 2006; Sillett *et al*., 2001; Shaw *et al*., in press a), and the marine estuarian fish, *Fundulus heteroclitus* (killifish, mummichog) (Stanton *et al*., 2006; Shaw *et al*., in press b).

Genomics and other molecular tools can be used in these species to examine the biological effects of low level environmental contamination. In *Daphnia*, for example, we have observed several important patterns of response that directly inform the ways in which such data can be used. First, we observed significant alterations in patterns of gene expression in response to low, environmentally relevant concentrations of several metals of toxicological concern (arsenic, cadmium, zinc, silver), demonstrating that genomics tools can be used to investigate such low level exposures in intact animals that are ecologically relevant (Shaw *et al*., 2006; Sillett *et al*., 2001; Shaw *et al*., in press a). Second, we observed distinct patterns of gene alteration that were specific to each toxicant, suggesting that such agent-specific "fingerprints" of response can be used to evaluate both individual toxicants of concern as well as potentially elucidating the contributions of individual components in complex mixtures (Shaw *et al*., 2006; Shaw *et al*., in press a). Third, we observed changes in genes that are directly relevant to the toxicological and biological mechanism of action of each agent, some of which confirmed previous observations and some of which led to new hypotheses (Shaw *et al*., 2006; Sillett *et al*., 2001; Shaw *et al*., in press a). In the latter regard, for example, we observed patterns of response indicating that arsenic and cadmium each act as potent endocrine disruptors in *Daphnia*, and likely, other aquatic invertebrates. Fourth, genomics provides a means of identifying specific genes that are of potential utility as biomarkers of specific exposures (Shaw *et al*., 2006; Sillett *et al*., 2001; Shaw *et al*., in press a). Finally, we observed patterns of gene response that suggested new ways of analyzing such complex data sets, and these methods, in turn, suggest important biological principles that inform us as to how to interpret such molecular level responses in terms of physiological and patho-physiological response (Shaw *et al*., in press a).

In particular, we discovered that parametric techniques provide a poor means of analyzing gene responses among a population of individuals, at least for most genes that were altered (Hamilton, 2006; Shaw *et al*., in press a). Specifically, widely used parametric methods such as SAM (Statistical Analysis of Microarrays, essentially a t-test comparing control and experimental values for each gene) fail to detect most genes that appear to be altered, and are particularly poor at detecting genes that are down-regulated rather than up-regulated by a given stimulus. Yet, graphical representations of the data clearly indicated that such genes were being altered, and that more genes were being down-regulated than up-regulated at environmentally relevant, low levels of toxicant exposure. Moreover, the same genes were altered in the same direction in every individual in the experimental group, albeit to different absolute levels of expression. Based on these observations, we developed several non-parametric methods for analyzing altered gene expression, the most accurate of which employs a rank-based approach using the median expression values, which we call Qualitative Expression Difference analysis or QED (Hamilton, 2006; Shaw *et al*., in press a).

Use of such nonparametric methods then led us to consider whether this was an accurate reflection of the underlying molecular processes we are measuring, and what might be the biological basis for such qualitative differences in steady-state mRNA expression (Hamilton, 2006; Shaw *et al*., in press a). There are several well-known mechanisms that can explain such qualitative versus strictly quantitative alterations in gene expression. First, it is well understood that natural populations — and even highly inbred experimental animal populations, such as inbred strains of laboratory mice – exhibit a wide inter-individual variation for any given quantitative parameter. For example, all animals in a population might respond to exposure to a given drug or toxicant by inducing expression of certain Phase I and Phase II metabolic enzymes in their liver, but the absolute level of induced expression will vary across a fairly wide range among individuals depending on dose, age, sex, time after treatment, diet and other variables. Second, many genes are regulated only loosely at the mRNA level in terms of their absolute level of expression. For example, a gene product might be tightly regulated at the level of protein expression or enzyme activity. When a cell requires a higher level of expression of that protein, it may increase gene transcription but merely require a larger pool of mRNA to satisfy its protein needs but not tightly regulate the absolute level of that mRNA pool. Third, for many genes, the control or basal state level of a gene is highly variable, oscillating about a median value as the gene cycles off and on at some steady-state level of expression. However, with specific stimuli, such genes often are induced or suppressed to a much more tightly regulated level. In this case, although the experimental value might have a tight mean and standard deviation among individuals, the control value may be variable enough that parametric techniques will not detect the change in expression as significant. Fourth, one could have polymorphisms in the population such that some individuals exhibit a large alteration in expression of a given gene while other individuals have a smaller change or no change. Such groups will also display apparently large variability in absolute expression when comparing means, but might represent a significant underlying response to a particular stressor. For these reasons we favor using non-parametric techniques, alone or in combination with parametric techniques, for examining alterations in gene expression. Determining which genes behave in parametric or non-parametric fashion also provides insight into the underlying biology of each gene and its regulation in response to environmental stimuli (Hamilton, 2006; Shaw *et al.*, in press a).

Genomics and its sister methodology, proteomics, provide powerful tools for examining subtle and specific responses to low levels of environmental toxicants and other environmental stressors, and might be particularly useful in the marine environment where it is more typical that multiple stressors come together in concert to produce complex biological and pathological responses (Hamilton, 2006). Selection of appropriate species will be important and involves weighing many factors, but key among them are that the species should ideally be both a keystone and sentinel species for its habitat; it should adequately represent specific habitats of concern, e.g., estuarian versus benthic, etc.; it should ideally be amenable to both field and laboratory study; and it should have or be amenable to developing the appropriate genomic tools including a sequenced genome at the gene or expressed mRNA level and ideally the availability of affordable, full genome microarrays. *Daphnia* is a useful freshwater aquatic model that satisfies these criteria (Shaw *et al.*, 2006; Sillett *et al.*, 2001; Shaw *et al.*, in press a). The estuarine euryhaline teleost, *Fundulus heteroclitus* (killifish) satisfies most of these initial criteria, and both a sequenced genome and availability of microarrays will soon be accomplished for this important model species (Stanton *et al.*, 2006; Shaw *et al.*, in press b). Other marine species should be proposed and developed as model marine organisms for genomic and toxicogenomic studies based on consensus in order to fulfill other research needs or to represent specific ecological niches of interest.

One area of particular interest to us is the adaptive response in relation to environmental toxicant exposures. There are many different levels of potential adaptive response representing different levels of cost and outcome. The optimal adaptive response is one that is fully successful with no cost, but this is likely to be a rare occurrence. The worst case scenario for an individual or population is to have an unsuccessful or only partially successful adaptive response that has a great cost. Other possible outcomes lie between these two extremes and likely represent most responses to environmental stressors at typical exposure levels. Genomics provides a means for examining both the gene level basis for adaptive responses, providing insight into possible mechanisms, but also provides insight into the possible outcomes for the adaptive response and its long-term consequence. Using *Daphnia* as a model and adaptation to elevated levels of swim water cadmium as an acute and/or chronic stressor, we evaluated the response of animals at the genomic, physiological and population level (Shaw *et al.*, 2006). As expected, both short- and long-term exposure to cadmium induced expression of metallothionein and other cadmium- and zinc-responsive genes. Long-term exposure to low levels led to the development of an adapted population of *Daphnia* that was highly resistant to acute, high-dose cadmium exposures compared to naïve controls. However, reproductive success was compromised in these animals as one cost of this adaptation in clean or low dose cadmium water, whereas these animals had higher survival and reproductive success at higher cadmium levels. Return of animals to clean water led to a rapid (within days) return to a control phenotype, and these animals responded similarly to naïve animals to a second challenge, suggesting that this adaptive response is highly transient, plastic, and requires continual stressor exposure to maintain (Shaw *et al.*, 2006; Shaw *et al.*, in press a). Concordance of molecular, physiological and population data across these experiments suggests the value of using the more sensitive genomic level responses to examine the adaptive response and other environmental responses to stressors in the natural marine environment at extremely low levels of exposure, perhaps well below those levels or at much earlier time points than are required to see such responses at the pathophysiological or population levels.

Toxicity vs. hormesis in evaluating health effects: applications to bioassays using marine organisms

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An unexplored, yet challenging subject in ecological risk assessment may be ascribed to the impact of aquatic contaminants at subtoxic concentration ranges. Background is provided by the studies of hormesis (or positive stimulation of biological events) in bioassay studies in view of a predictive evaluation of pollutant toxicity to environmental health. When running bioassays, dose- or concentration-related effects imply acute toxicity at the highest tested agent (or mixture) levels, whereas exposures to low agent levels have been associated to either linear or to threshold trends, accordingly regulated in the legislation for xenobiotics and physical agents. Aside to these established criteria, an extensive body of literature has reported that dose- or concentration-related trends for radiation and chemicals show a low-level interval where the adverse effects – observed at higher agent levels – are reverted to a positive stimulation of the biological events being evaluated including, e.g., increases in growth rate, fertilization success, or enzyme activities. This stimulation is currently termed hormesis, and its relevance has been raised in a number of disciplines, such as radiobiology, carcinogenesis, and toxicology/pharmacology. Hormesis has been reported in a number of organisms, in vitro and cell-free systems, also including several bioassays utilizing marine biota, from the pioneering studies by Stebbing (1979) utilizing hydroid coelenterates up to recent papers based on sea urchin bioassays (De Nicola *et al*., 2004; in press). The extent and relevance of the hormesis phenomenon have been reviewed by Calabrese and his co-workers (2005), and a review of the literature in the last 25 years shows a dramatic increase of papers referring to hormesis, from scanty publications until 1995 up to approximately 400 papers cited in MedLine from 2001 to August 2006. The evaluation of concentration-related shifts from hormesis to toxicity requires adequate design in bioassays, including: a) broadly ranging agent concentrations, and b) adequate definition of controls. Concentration intervals should be designed in a realistic range above background contaminant levels, yet below the classical “no-adverse-effect-level” (NOAEL), that may conceal the onset of hormetic effects provided that adequately low agent concentrations are tested. If bioassays are to measure parameters as growth rate, or enzyme activity, untreated controls provide values that may either be increased (hormesis) or decreased (toxicity) as a function of agent levels. Otherwise, a number of parameters may range along with an event frequency, such as 0% to 100%. In this case, assigning optimal values to controls will prevent from assessing any hormetic effect when compared to a “perfect” control value. Thus, bioassays relying on event frequencies should not assign optimal values in control schedules, whereas sub-optimal control values may allow us to assess hormetic effects, if any. Based on the experience of bioassay studies, the relevance of hormesis in the “real world” – e.g. in coastal water and sediment - may be envisioned. Beyond the limited scope of bioassays, the major relevance of hormesis in environmental health and risk assessment relies on the consequences of exposures to subtoxic levels of contaminants in confined water bodies (e.g. enclosed bays), in dilution ranges of contaminants that may exert hormetic activity. As far as some biota may thrive as an effect of hormesis, the ensuing population burst for the affected biota may result in a set of imbalances at the community level. Thus, targeted field studies might detect possible hormetic effects of low-level contaminants leading to selective changes in population densities for some species with detrimental consequences to other biota.

HORMESIS: DEFINITION AND HISTORICAL BACKGROUND

A growing body of literature in toxicology, pharmacology and radiobiology reports on hormesis, a phenomenon that may be defined as a positive stimulatory effect exerted by low concentrations, or doses, of agents that cause inhibitory or toxic effects when administered at increasing levels. Far from being an extravagant statement, the observed shifts from stimulatory ("hormetic") effects to inhibition and toxicity may be ascribed back to the Ancient Greek term *pharmakon*, with the opposite meanings of «poison» and of «medicinal agent». A first pioneering study by Hugo Schulz (portrayed in Figure 1) in 1888 was conducted to test a theory proposed by Rudolph Arndt, reporting on the effects of several toxicants in yeast cultures, showing that high level-associated toxicity was reverted to growth stimulation when yeast was exposed to low agent levels (Schulz, 1888). Thereafter this phenomenon was also termed "Arndt-Schulz effect", and numerous reports were published in the early decades of the 20th century (reviewed by Calabrese and Baldwin, 2000a,b). A renewed interest in the studies of hormesis has been attributed to the early reports by Anthony Stebbing and his co-workers (1976; 1979; 1982) that opened a re-appraisal and still-thriving investigations on hormesis in environmental sciences and in other disciplines (reviewed by Calabrese, 2005; Calabrese and Blain, 2005). The growing awareness of this phenomenon in the scientific community, however, has failed as yet to result in any consequences in terms of actions by either environmental protection or drug regulatory agencies (van der Schalie and Gentile, 2000; Calabrese, 2001; Kitchin and Drane, 2005; Mundt, 2006).

Fig. 1. Hugo Paul Friedrich Schulz (1853-1932).

HORMESIS: A BROAD RANGING PHENOMENON

Evidence for the growing interest in hormesis is given in Figure 2, showing the steady increase of MedLine-archived publications (updated August 2006) displaying the term "hormesis" in their titles and/or abstracts. Over 730 similar citations were retrieved from Web of Science as compared to the 406 found in PubMed (1956-2006).

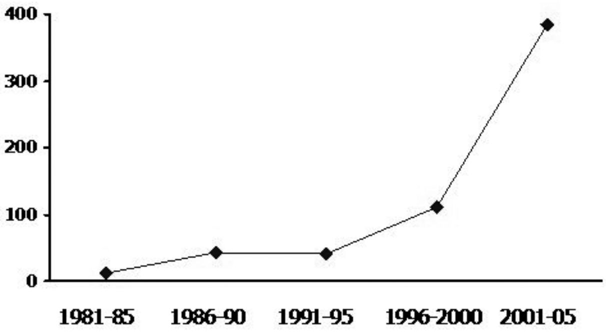
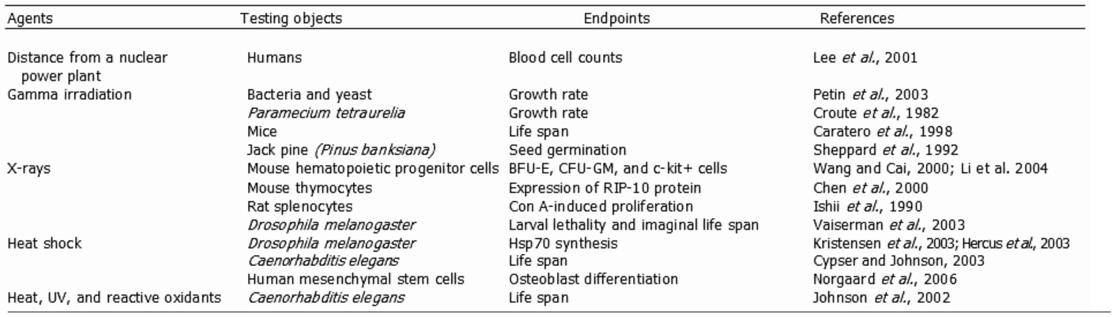


Fig. 2. Production of journal articles including the term "hormesis" as retrieved by MedLine (August 2006).

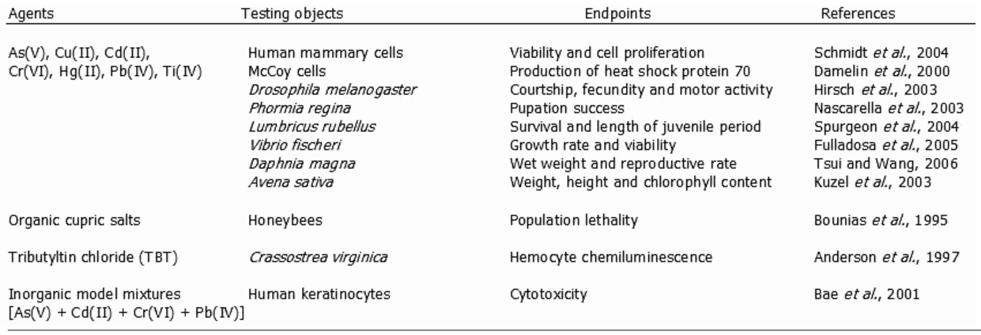
The literature records include physical agents, inorganics, a diversified range of organics (including pesticides, solvents, pharmaceuticals, and carcinogens), and complex mixtures, as shown in Tables 1a to 1d. The organisms where hormetic effects were assessed include a broad range of phyla, from bacteria and microalgae up to higher plants and mammals. The endpoints evaluated in hormesis studies mostly focused on cell growth, yet a number of other parameters were evaluated, including organismal, cellular and molecular endpoints, such as life span, success in early development, cell differentiation, and protein expression and regulation (Tables 1a, b, c, d).

Table 1a. Selected hormesis-related literature from MedLine (August 2006): physical agents.



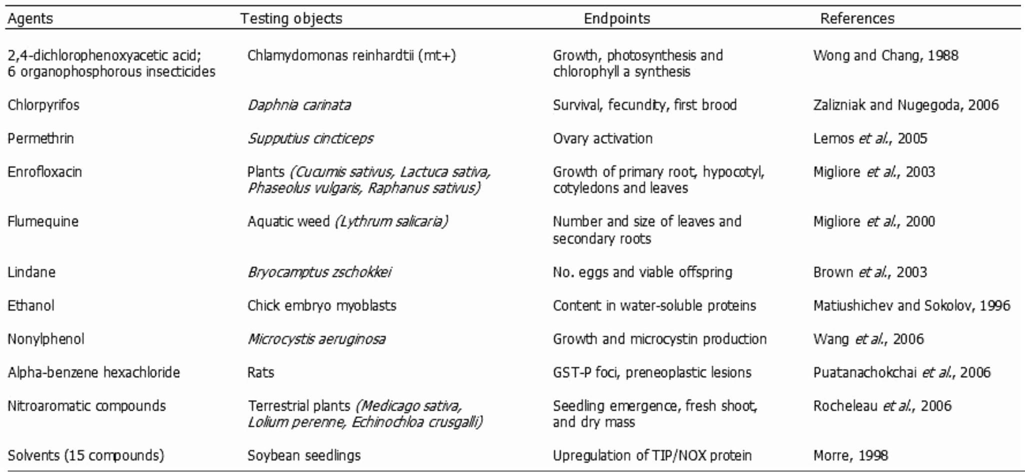
Agents	Testing objects	Endpoints	References
Distance from a nuclear power plant	Humans	Blood cell counts	Lee *et al.*, 2001
Gamma irradiation	Bacteria and yeast	Growth rate	Petin *et al.*, 2003
	Paramecium tetraurelia	Growth rate	Croute *et al.*, 1982
	Mice	Life span	Caratero *et al.*, 1998
	Jack pine *(Pinus banksiana)*	Seed germination	Sheppard *et al.*, 1992
X-rays	Mouse hematopoietic progenitor cells	BFU-E, CFU-GM, and c-kit+ cells	Wang and Cai, 2000; Li et al. 2004
	Mouse thymocytes	Expression of RIP-10 protein	Chen *et al.*, 2000
	Rat splenocytes	Con A-induced proliferation	Ishii *et al.*, 1990
	Drosophila melanogaster	Larval lethality and imaginal life span	Vaiserman *et al.*, 2003
Heat shock	*Drosophila melanogaster*	Hsp70 synthesis	Kristensen *et al.*, 2003; Hercus *et al.*, 2003
	Caenorhabditis elegans	Life span	Cypser and Johnson, 2003
	Human mesenchymal stem cells	Osteoblast differentiation	Norgaard *et al.*, 2006
Heat, UV, and reactive oxidants	*Caenorhabditis elegans*	Life span	Johnson *et al.*, 2002

Table 1b. Selected hormesis-related literature from MedLine (August 2006): inorganics.



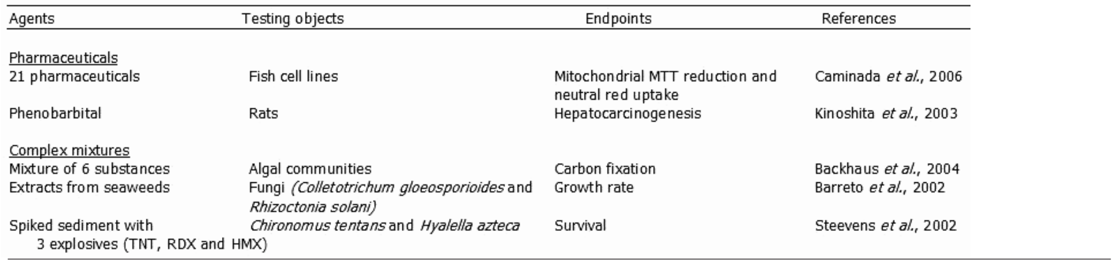
Agents	Testing objects	Endpoints	References
As(V), Cu(II), Cd(II), Cr(VI), Hg(II), Pb(IV), Ti(IV)	Human mammary cells	Viability and cell proliferation	Schmidt *et al.*, 2004
	McCoy cells	Production of heat shock protein 70	Damelin *et al.*, 2000
	Drosophila melanogaster	Courtship, fecundity and motor activity	Hirsch *et al.*, 2003
	Phormia regina	Pupation success	Nascarella *et al.*, 2003
	Lumbricus rubellus	Survival and length of juvenile period	Spurgeon *et al.*, 2004
	Vibrio fischeri	Growth rate and viability	Fulladosa *et al.*, 2005
	Daphnia magna	Wet weight and reproductive rate	Tsui and Wang, 2006
	Avena sativa	Weight, height and chlorophyll content	Kuzel *et al.*, 2003
Organic cupric salts	Honeybees	Population lethality	Bounias *et al.*, 1995
Tributyltin chloride (TBT)	*Crassostrea virginica*	Hemocyte chemiluminescence	Anderson *et al.*, 1997
Inorganic model mixtures [As(V) + Cd(II) + Cr(VI) + Pb(IV)]	Human keratinocytes	Cytotoxicity	Bae *et al.*, 2001

Table 1c. Selected hormesis-related literature from MedLine (August 2006): pesticides and other organics.



Agents	Testing objects	Endpoints	References
2,4-dichlorophenoxyacetic acid; 6 organophosphorous insecticides	Chlamydomonas reinhardtii (mt+)	Growth, photosynthesis and chlorophyll a synthesis	Wong and Chang, 1988
Chlorpyrifos	*Daphnia carinata*	Survival, fecundity, first brood	Zalizniak and Nugegoda, 2006
Permethrin	*Supputius cincticeps*	Ovary activation	Lemos *et al.*, 2005
Enrofloxacin	Plants *(Cucumis sativus, Lactuca sativa, Phaseolus vulgaris, Raphanus sativus)*	Growth of primary root, hypocotyl, cotyledons and leaves	Migliore *et al.*, 2003
Flumequine	Aquatic weed *(Lythrum salicaria)*	Number and size of leaves and secondary roots	Migliore *et al.*, 2000
Lindane	*Bryocamptus zschokkei*	No. eggs and viable offspring	Brown *et al.*, 2003
Ethanol	Chick embryo myoblasts	Content in water-soluble proteins	Matiushichev and Sokolov, 1996
Nonylphenol	*Microcystis aeruginosa*	Growth and microcystin production	Wang *et al.*, 2006
Alpha-benzene hexachloride	Rats	GST-P foci, preneoplastic lesions	Puatanachokchai *et al.*, 2006
Nitroaromatic compounds	Terrestrial plants *(Medicago sativa, Lolium perenne, Echinochloa crusgalli)*	Seedling emergence, fresh shoot, and dry mass	Rocheleau *et al.*, 2006
Solvents (15 compounds)	Soybean seedlings	Upregulation of TIP/NOX protein	Morre, 1998

Table 1d. Selected hormesis-related literature from MedLine (August 2006): miscellaneous.



Agents	Testing objects	Endpoints	References
Pharmaceuticals			
21 pharmaceuticals	Fish cell lines	Mitochondrial MTT reduction and neutral red uptake	Caminada *et al.*, 2006
Phenobarbital	Rats	Hepatocarcinogenesis	Kinoshita *et al.*, 2003
Complex mixtures			
Mixture of 6 substances	Algal communities	Carbon fixation	Backhaus *et al.*, 2004
Extracts from seaweeds	Fungi *(Colletotrichum gloeosporioides* and *Rhizoctonia solani)*	Growth rate	Barreto *et al.*, 2002
Spiked sediment with 3 explosives (TNT, RDX and HMX)	*Chironomus tentans* and *Hyalella azteca*	Survival	Steevens *et al.*, 2002

Most of this literature is confined to testing individual agents by single species bioassays. However, efforts have been devoted to gain information on hormetic effect by testing model or complex mixtures in multi-species studies, as e.g. in the report by Backhaus *et al*. (2004) who tested the effects of mixtures in algal communities. Studies at the microcosm or at the ecosystem level are not reported, to the best of our knowledge, and await appropriate design and implementation.

HORMETIC EFFECTS IN MARINE ORGANISMS

Hormetic effects have been reported in marine bacteria, yeast, micro- and macroalgae, coelenterates, polychaetes, bivalves, echinoderms, and fishes, as shown in Table 2. The agents displaying hormetic effects in marine biota include inorganics, metallorganic compounds (TBT), and several organics as pharmaceuticals and complex mixtures. The recorded endpoints included growth rate, reproductive and developmental success, and tissue regeneration (Table 2). Chemiluminescence methods have been utilized, e.g. hemocyte activation in bivalves, and luminol-dependent chemiluminescence in sea urchin gastrulae, as shown in Figure 3 (Pagano *et al*., 2001a,b).

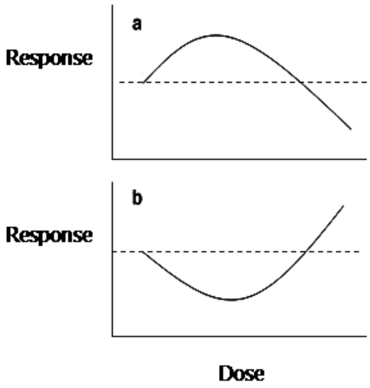
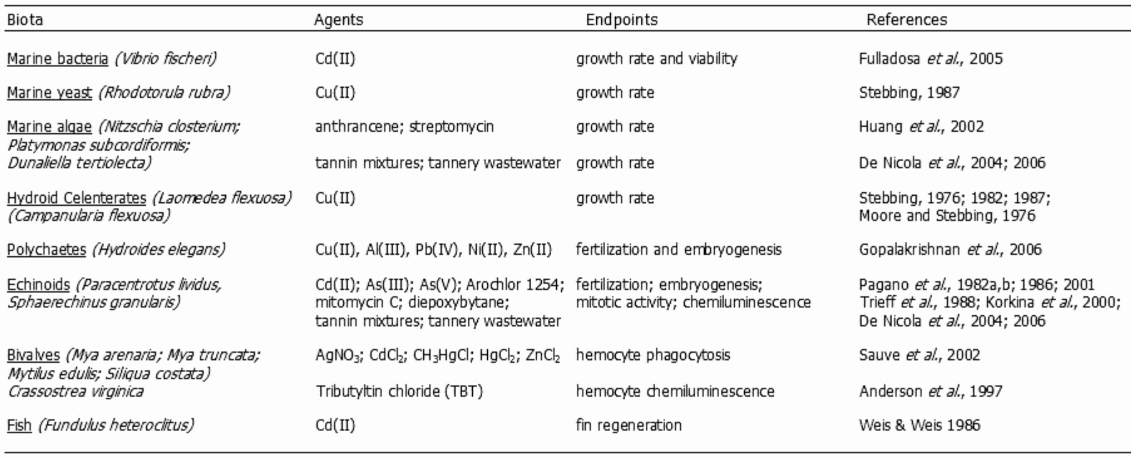


Fig. 3. The most common form of the hormetic dose-response curve depicting low-dose stimulatory and high-dose inhibitory responses. A. the β- or inverted U-shaped curve. B. the hormetic dose-response curve depicting low-dose reduction and high-dose enhancement of adverse effects, the J- or U-shaped curve (from Calabrese, 2004).

Table 2. Bioassay reports on hormesis utilizing marine organisms.



Biota	Agents	Endpoints	References
Marine bacteria *(Vibrio fischeri)*	Cd(II)	growth rate and viability	Fulladosa *et al.*, 2005
Marine yeast *(Rhodotorula rubra)*	Cu(II)	growth rate	Stebbing, 1987
Marine algae *(Nitzschia closterium;* *Platymonas subcordiformis;*	anthrancene; streptomycin	growth rate	Huang *et al.*, 2002
Dunaliella tertiolecta)	tannin mixtures; tannery wastewater	growth rate	De Nicola *et al.*, 2004; 2006
Hydroid Celenterates *(Laomedea flexuosa)* *(Campanularia flexuosa)*	Cu(II)	growth rate	Stebbing, 1976; 1982; 1987; Moore and Stebbing, 1976
Polychaetes *(Hydroides elegans)*	Cu(II), Al(III), Pb(IV), Ni(II), Zn(II)	fertilization and embryogenesis	Gopalakrishnan *et al.*, 2006
Echinoids *(Paracentrotus lividus,* *Sphaerechinus granularis)*	Cd(II); As(III); As(V); Arochlor 1254; mitomycin C; diepoxybytane; tannin mixtures; tannery wastewater	fertilization; embryogenesis; mitotic activity; chemiluminescence	Pagano *et al.*, 1982a,b; 1986; 2001 Trieff *et al.*, 1988; Korkina *et al.*, 2000; De Nicola *et al.*, 2004; 2006
Bivalves *(Mya arenaria; Mya truncata;* *Mytilus edulis; Siliqua costata)*	$AgNO_3$; $CdCl_2$; CH_3HgCl; $HgCl_2$; $ZnCl_2$	hemocyte phagocytosis	Sauve *et al.*, 2002
Crassostrea virginica	Tributyltin chloride (TBT)	hemocyte chemiluminescence	Anderson *et al.*, 1997
Fish *(Fundulus heteroclitus)*	Cd(II)	fin regeneration	Weis & Weis 1986

An example of hormesis in marine organisms exerted by complex mixture may be provided by our recent studies of vegetable and synthetic tannins utilized in leather tanning industry, and of tannery wastewater in sea urchin *(Paracentrotus lividus* and *Sphaerechinus granularis)* early development and in growth rate of marine and freshwater microalgae *(Dunaliella tertiolecta* and *Selenastrum capricornutum)*. Individual tannin compounds were reported to display a hormesis to inhibition shift, as e.g. tannic acid and gallic acid, as shown in Table 3. In our studies, vegetable tannin from *Acacia* sp., synthetic phenol-based tannin, and leather tannery wastewater shared hormetic effects at comparable tannin concentrations. As shown in Figure 4, fertilization rate of sea urchin sperm displayed an increase vs. controls at low tannin levels. The same applied to the frequencies of developmental defects in tannin-exposed sea urchin embryos, as shown in Figure 5. As discussed below, the hormesis/toxicity shift reached statistical significance provided that control quality was set at suboptimal frequencies of developmental defects, whereas the assignment of optimal control quality either displayed a similar, yet non-significant trend, or turned to monotonic concentration-related trends.

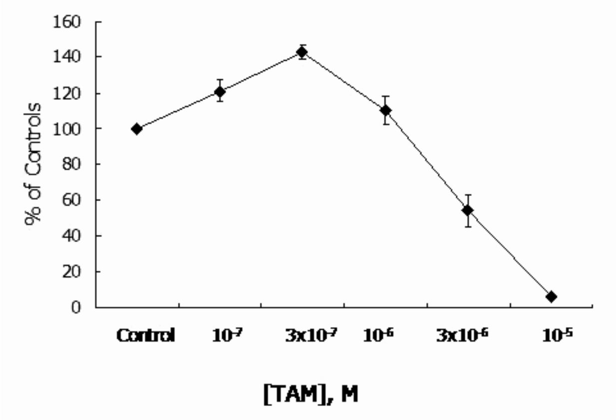


Fig. 4. Luminol-dependent chemiluminescence in sea urchin *(Paracentrotus lividus)* gastrulae exposed to tamoxifen (from Pagano *et al.*, 2001a).

Table 3. Selected information on tannin-related toxic and hormetic effects.

Agents	Effects	References
Camellin B	induced apoptosis in HeLa cell line	Wang *et al.*, 2001
Hypericum perforatum extract & oil	↑ immunostimulating activity in rats ↑ immunosuppressing activity	Anonymous, 2001
Gallic acid	non-toxic up to 5 g/kg body weight in mice	Rajalakshmi *et al.*, 2001
Areca nut polyphenols and tannin	oral cancer promotion	Jeng *et al.*, 2001
Terminalia arjuna tannin extract	↓ 2AF –induced mutagenicity	Kaur *et al.*, 2000
Tannic acid	↑ metabolic activation of a few mutagens anticlastogenic and antimutagenic effects	Chen and Chung, 2000 Sasaki *et al.*, 1990
Tannins	↑ inhibitory activity on lipid peroxidation	Hong *et al.*, 1995

PLANNING HORMESIS-ORIENTED STUDIES

Concentration range

In most bioassay reports, agent concentrations, or doses range from NOAEL to acute toxicity or lethal effects. Within this range, two types of trends are commonly recognized, i.e.: a) threshold trends, characterized by a critical concentration/dose superimposable with NOAEL and above which adverse effects are detected, and b) linear trends, assumed by most agencies for radiation, genotoxins and carcinogens, deemed to exert decreasing, yet definite damage even at very low-level exposures.

In the presence of these established criteria, hormetic effects may arise from serendipitous observations and, even so, may be disregarded as "data fluctuations" below threshold or at very low agent levels. Additional factors in failure to recognize hormesis may be attributed to a widespread unawareness of this phenomenon, and/or to a form of discomfort to report an *improvement* of an adverse event for low levels of an agent exerting damages when administered at higher levels.

Beyond this widespread attitude and practice, focusing studies on possible hormetic effects both requires open-minded workers and, especially, demands a study design that shall encompass a broad concentration range that must include low agent levels, e.g., for merely practical purposes, by three to five orders of magnitude below threshold (Calabrese and Blain, 2005).

Also advised is to plan suitable numbers of subjects in animal studies for low-level exposures fitting the requirement to improve the statistical power of data, as suggested by Hunt (2002).

A crucial issue: control quality

Whenever the bioassay endpoint is quantified by e.g. measuring enzyme activities, growth rate or any other number that may be attributed to "normal", or control values, then inhibition and, respectively, hormesis will be associated with lower or higher values compared to controls. On the other hand, defining suitable controls in detecting hormetic effects may be viewed as a *sine qua non* in all those bioassays whose endpoints are measured by relative frequencies, e.g. expressed by decimal or percent values. In these bioassays, a broadly shared prejudice claims that controls should be characterized by 100% of a physiological event (e.g. fertilization, or hatching success), and by zero value for adverse events (e.g. malformations or other abnormalities). This criterium, by definition, hides any hormetic effect, as no improvement of performance can be detected vs. controls, once control quality has been established up to optimal values. In the case of fertilization success, establishing control values at 100% may conceal an additional pitfall, as 100% fertilization may actually hide a theoretical (and impossible) higher-than-optimal fertilization rate due to excess sperm cells compared to the available ovocytes. Hence, in this case one is both unable to assess any hormetic effect (fertilization rate cannot exceed 100%) and a loss of test sensitivity also occurs vs. inhibitory effects, as spermiotoxicity may only be detected once excess sperm are inactivated. In this kind of bioassay, control quality should be defined *below* optimal values, e.g. by setting control values at values closer to a "central" performance (e.g. 50 to 70% for physiological events), thus allowing for the observation of both adverse outcomes – without any sensitivity bias – and by enabling the observation of hormetic effects. As shown in Figure 5, fertilization rate of sea urchin sperm was either enhanced or inhibited provided that fertilization rate was established at suboptimal values.

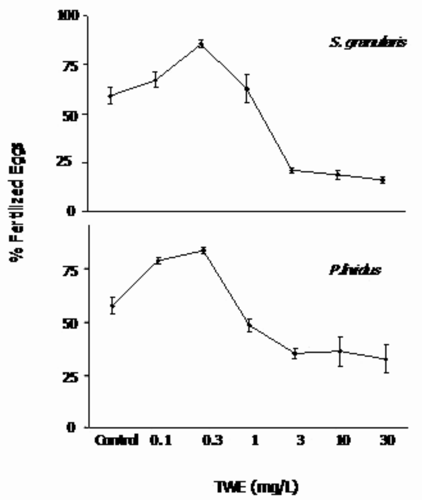


Fig. 5. Fertilization rate of sea urchin *(Paracentrotus lividus and Sphaerechinus granularis)* sperm exposed to tannin water extract (TWE) (from De Nicola *et al.,* 2004).

As an additional, yet not neglectable argument, one might note that optimal control quality may, or may not reflect the actual situation in natural populations, where the occurrence of adverse events may be higher than zero and physiological events may be far from a 100% performance.

Therefore, both on the basis of experimental evidence and of common sense considerations (of mathematical and of ecological nature), one may recommend to avoid relying on unrealistic and illogical criteria as those attributing an optimal performance to control cultures.

RELEVANCE OF HORMESIS AMONG ENVIRONMENTAL HEALTH EFFECTS

The occurrence – or the relevance - of hormesis has been debated stating that this phenomenon cannot be generalized nor can be utilized as a default assumption in toxicology (Kitchin and Drane, 2005). A recent Staff Paper issued by the US EPA (March 2004) stated that as “the purpose of a risk assessment is to identify risk (harm, adverse effect, etc.), effects that appear to be adaptive, non-adverse, or beneficial may not be mentioned”, as discussed by Mundt (2006).

Several arguments may be raised against this attitude (Calabrese, 2004). Hormesis is currently associated with the onset of “beneficial” effects, a definition that has mostly derived from studies of individual agents tested in single-organism bioassays. However, this definition may disregard some effects of hormesis as they may especially apply to aquatic ecosystems. In spite of the current scarcity of reports on hormesis in community studies (Backhaus *et al*., 2004) and in multi-species bioassays (Barreto *et al*., 2002; De Nicola *et al*., 2004; in press; Huang *et al*., 2002; Migliore *et al*., 2003; Steevens *et al*., 2002) one may anticipate that hormetic effects may be detected at the community level, provided that *ad hoc* studies are carried out. In other terms, if bioassay reports assessing the toxic effects of xenobiotics were later confirmed in exposed communities, one may wonder why the hormetic effects of some defined xenobiotics should not be observed in microcosms or at the community level. Provided that hormetic effects, at a given agent level, may involve some components of the community, the outcome may imply excess population growth for some species at the expenses of other biota, in analogy with the well-established effects of excess nutrient levels (Occhipinti-Ambrogi *et al*., 2005). Hence, a “non-adverse, or beneficial“ effect for some species may result in overall perturbations of often fragile community balances.

Another aspect of possible adverse effects induced by low-level toxicants may be exemplified by the fertilization success of sea urchin (*Echinus esculentus*) sperm exposed to cadmium or zinc salts administered separately or in equimolar mixtures (Pagano *et al*., 1986). As shown in Figure 6, a shift from hormesis to inhibition of fertilization success was exhibited by each of the agents in the micromolar range, whereas a dramatic spermiotoxic effect was exerted by the model mixture of Cd(II) + Zn(II) in the same concentration range. This early result may point to an as yet poorly explored subject, i.e. concentration-dependent trends by model mixtures and, even more so, by complex mixtures of agents that exert hormesis/toxicity shifts when administered individually.

Together, background laboratory experience should prompt new investigations focused on the potential adverse effects of hormesis – especially by complex mixtures - at the ecosystem level.

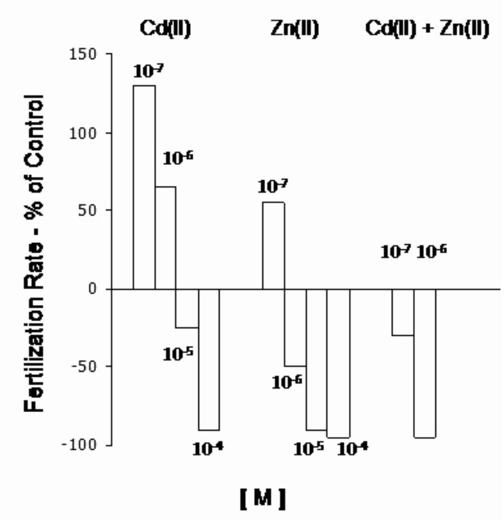


Fig. 6. Hormesis/inhibition shift in fertilization success of *Echinus esculentus* sperm exposed to CdCl$_2$, ZnCl$_2$, or their equimolar mixture (from Pagano *et al.,* 1986).

PROSPECTS IN ECOLOGICAL RISK ASSESSMENT

As stated by Chapman (2001), “a major research need is the extension of hormesis beyond chemical stressors to abiotic (e.g., habitat) and biotic stressors (e.g., species introductions, organism interactions). An overreaching research need is to determine for all stressors with model organisms, populations, and communities whether hormesis has positive, neutral, or adverse effects“. To date, the phenomenon called hormesis has been broadly documented, yet confined to bioassay studies. Some opinions point to hormesis as a generalized phenomenon, resulting in “stimulatory” or “beneficial” effects (Calabrese, 2004); yet we still ignore to what extent individual xenobiotics or mixtures do – or do not – exert hormetic effects, provided that adequate studies are designed. Even less we know about the extent low-level agents exert beneficial, no, or adverse effects at the ecosystem scale. The answers to these open questions are most likely diversified according to the xenobiotics or mixtures to be investigated. However, even in the case of a real advantage taken by low-level agents in a community, this advantage may be reasonably confined to some biota and not to others, hence resulting in uncontrolled alterations in the ecosystem. Novel research avenues are open in elucidating these amazing environmental problems.

Biomarkers in human and environmental health risk assessment

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SUMMARY

Marine pollution is a major threat to human and environmental health. This short paper describes how some recent technological advances in medical diagnostics and molecular biology are being adapted and applied to study the impacts of contamination in the marine environment. The development of biomarkers of increasing sophistication can provide a means of integrating human and environmental health risk assessment and providing a more balanced view of the threat posed to humans and to wildlife by contamination of the marine environment.

INTRODUCTION: MARINE POLLUTION AND HEALTH

Marine pollution is a major threat to human and environmental health. There is growing concern that chemical, physical and biological agents present in the marine environment may cause or otherwise influence the onset of various illnesses or disorders in susceptible human and wildlife populations. Health impacts arise due to the intake of food or water contaminated with chemicals or harmful micro-organisms, exposure to adverse weather conditions, air pollution and general industrial pollution and to the insidious effects of long term, simultaneous exposure to low levels of complex mixtures of environmental pollutants. More than 100,000 chemicals in current use are ultimately discarded into the environment, the majority of which will not have been tested for potential toxic effects on humans or wildlife. Whilst initiatives such as EU REACH will begin to address this deficiency, relatively little attention is being paid to the more subtle relationships between the structure and function of natural and contaminated ecosystems and human health, or to the value of biodiverse ecosystems in maintaining human well-being and health (the biophilia concept).

THE RISK ASSESSMENT PROCESS

One of the difficulties in forming balanced judgements of the impacts of marine pollution on human and wildlife species is that most human and ecological risk assessments are conducted separately. Although both human and ecological risk assessment protocols have a common goal, ‘to provide a quantitative estimate of the probability of an adverse effect occurring as a consequence of a contaminant release’ (Newman, 1998), there are differences in regulatory practise, emphasis and technical feasibility that lead to differences in the endpoints commonly considered. Human health risk assessment is concerned solely with the protection of human health and is focused on the individual, whilst ecological risk assessment must also consider the consequences for populations, communities and ecosystems (see also Depledge, this volume). The impact at the individual level has then been given less emphasis.

As we learn more about the molecular and cellular mechanisms that form the basis of biological processes, the sheer scale on which humans and other species, as diverse as fish, worms and snails, share genes and cellular mechanisms in common becomes apparent. A further consequence of this is that they may be equally susceptible to the common mechanisms of action of toxic chemicals. Toxicological damage at the level of molecules (DNA, protein and lipid), cells (integrity of membranes, function of subcellular organelles) and tissues (necrotic lesions, structural changes) may be remarkably similar between quite diverse species. Whilst consideration must always be given to exposure scenarios and to species differences in the patterns of uptake, metabolism and excretion, biomarkers (defined here as functional measures of exposure to stressors at the molecular, cellular or physiological level) can provide common, measurable endpoints to enable human and ecological risk assessments to be considered in a more integrated way.

BIOMARKERS

Biomarkers are generally classified into four major categories advocated by the National Research Council, (a) internal dose, (b) biologically effective dose, (c) early biological effects and (d) susceptibility and are illustrated in Figure 1. This describes a continuum from exposure to disease, although different classifications may of course overlap. For example, blood or tissue contaminant concentrations, or metabolites, may be used to estimate exposure; altered enzyme activities may indicate biologically effective doses, susceptibility of pre-clinical effects, whilst pathological lesions provide evidence of adverse consequences.

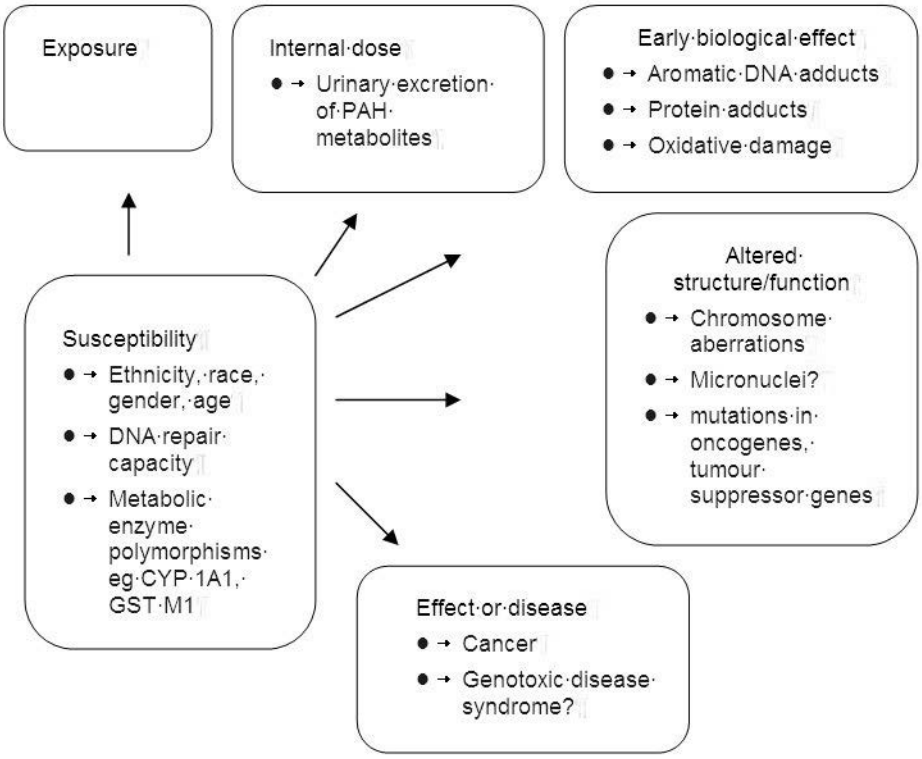

Fig. 1. The application of biomarkers as early endpoints in relation to exposure to polyaromatic hydrocarbons (PAHs).

Recent advances in molecular biology, diagnostic and analytical technologies mean that these techniques are increasingly becoming available for use with wildlife species, including mammals, fish and shellfish, with the promise of providing a more detailed insight into their toxicological and environmental responses to stressors. This has the potential to be hugely beneficial to the risk assessment, particularly of marine contaminated areas, as it addresses the lack of mechanistic detail and genetic sequence data which was previously available for the most popular invertebrate species used in aquatic ecotoxicology, such as the freshwater crustacean *Chironomus riparus,* the amphipod *Daphnia magna*, and the common mussel *Mytilus edulis*. Improved methods for determining exposure levels are also of particular relevance for assessing human exposure through contaminated food.

Among the novel biotools under development in our own laboratories for the assessment of marine pollution, we will describe in more detail the biomarkers of exposure to polyaromatic hydrocarbons.

POLYAROMATIC HYDROCARBONS

Polyaromatic hydrocarbons (PAHs) are persistent organic pollutants that occur ubiquitously as complex mixtures in both terrestrial and marine environments, occurring naturally in oil and gas deposits, as products of incomplete combustion and through the activities of man. They are of particular concern because of the very strong evidence of the carcinogenic and mutagenic properties of some compounds. In addition, PAHs can induce a broad spectrum of immunotoxic effects, leading to increased susceptibility to disease.

In general, PAHs are not particularly reactive and express little toxicity. Once ingested, however, their metabolic transformation yields reactive products capable of interacting with macromolecules including DNA. There is growing evidence that certain DNA adducts formed in this way can become fixed as mutations of oncogenes or tumour-suppressor genes. For this reason, the determination of PAH exposure is of particular importance in human risk assessment in the monitoring of workplace exposure and in molecular cancer epidemiology. Sensitive, validated biomarkers of exposure, effect and susceptibility to the adverse effects of PAH exposure have been developed (Perera and Weinstein, 2000); they are illustrated in Figure 1.

The wreckage of oil tankers and discharges from oil terminals cause marine pollution by crude oil, which contains significant quantities of PAHs. When crude oil is released into the sea, oil films or slicks can spread over large areas. The smaller hydrocarbons have the highest vapour pressure and volatilise, leaving the less volatile hydrocarbons to sink and associate with sediments. Residues of PAHs can therefore exist in sediments long after the original spill has dispersed from the surface, where they may contaminate bottom dwelling organisms, including many invertebrate species low in the food chain. Invertebrates lack the capacity for effective biotransformation found in higher vertebrates, and tend to bioaccumulate and bioconcentrate PAHs. These do not tend to biomagnify up the food chain, as fish and aquatic vertebrates can readily transform PAHs. On the other hand, fish, birds or mammals (including humans) who feed directly on species such as molluscs may consume significant amounts of PAHs. There has been some success in adapting and applying biomarkers of PAH exposure developed for humans to aquatic mammals and fish, but despite intensive research effort, the rapid estimation of PAH exposure and effect in aquatic invertebrate species (other than by tissue residue analysis) remains problematic.

URINARY PAH METABOLITES IN ENVIRONMENTAL MONITORING

Since PAHs comprise a mixture of compounds, biomarkers of exposure and internal dose can either target the whole group of PAH isomers or specific individual PAH compounds. Quantification of the hydroxy PAH metabolite 1-hydroxypyrene in urine has been used as a sensitive and robust biomarker of exposure in humans but until now, urine has not been considered as a practical sampling method for ecological studies.

Urine is a suitable matrix for analysis in humans as it can be collected non-invasively and is relatively free from the lipid and protein that may interfere with the detection of PAHs. It is possible to extract urine non-destructively from crabs such as the common shore crab *Carcinus maenas* by applying gentle pressure to the antennal gland bladder, causing urine to flow through the operculum of the antennal gland. As crabs are capable of metabolising PAHs to some extent, monitoring the metabolites of PAHs in crab urine represents a realistic means of monitoring their bioavailability.

Most PAH compounds exhibit fluorescent properties due to the delocalisation of electrons in the aromatic ring structure. Studies in our laboratories have identified characteristic and concentration dependent fluorescence spectra in the urine of pyrene-exposed crabs compared to unexposed crabs. Although the pathways of metabolic transformation of PAH compounds are not completely understood in crustaceans, some of these fluorescent metabolites have tentatively been identified as glucosides and sulphates. This fluorescence method has been field tested during an environmental monitoring programme in the Karmsund Strait, Norway where a pollution gradient of PAH sediment contamination is known to extend outwards from an aluminium smelter at Hogevard. Crabs collected from sites located at decreasing distances from the smelter were found to have significantly elevated level of urinary PAH metabolites in their urine. This method offers a viable, rapid and inexpensive alternative to tissue residue analysis for determining the exposure to PAHs of crabs and potentially other crustaceans. Although the profile of PAH metabolites is species specific and therefore not directly comparable between *C. maenas* and humans, this generic concept provides a valid endpoint for exposure studies.

Human pathogens in the marine environment - an ecological perspective

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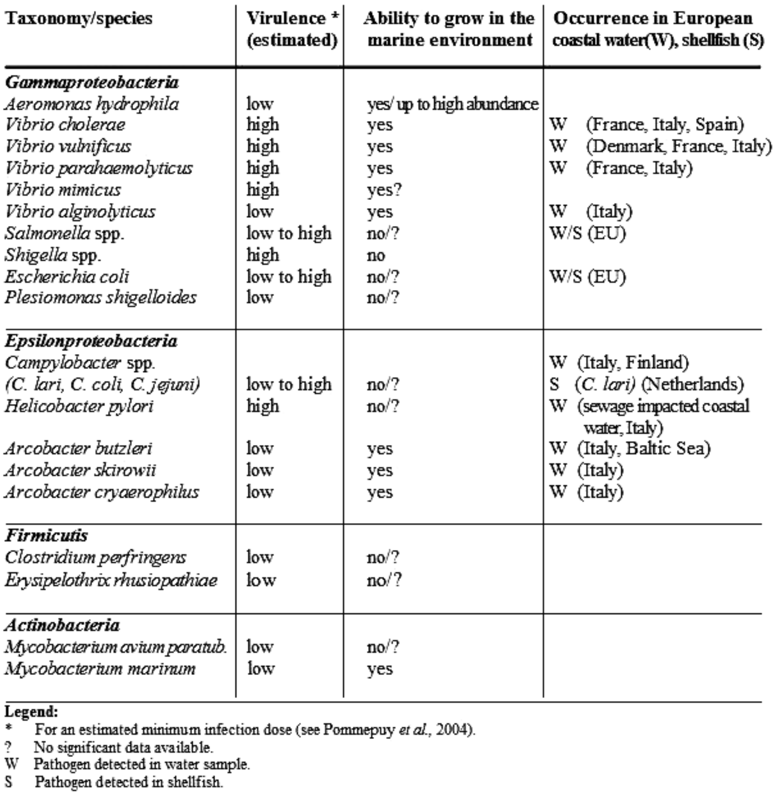
ABSTRACT

Pathogenic microorganisms in marine environments can present a major threat to human health with bathing and consumption of shellfish being the major transmission routes to humans (CIESM, 2004; Collins, 2003; Griffin *et al.,* 2003; Henrickson *et al.,* 2001; Yeung and Boor, 2004). A wide range of bacteria, viruses and protozoa are considered as pathogenic marine microorganisms causing a broad spectrum of human diseases. Most microorganisms have as main transmission mechanism the fecal-oral route, with sewage, run off, river discharge, groundwater seepaging, and sewage sludge disposals as major sources of microbial contamination of the marine environment. Survival and growth (the latter only relevant for some bacterial species) of pathogenic microorganisms in the marine environment are greatly influenced by human activities and environmental factors mainly triggered by man-made eutrophication. The health threat by pathogenic microorganisms in the marine environment is so far not well understood and not yet incorporated in a reliable monitoring scheme, especially since the detection of viruses, non-culturable bacteria and protozoa is greatly neglected. In conclusion, we suggest environmental management measures to decrease microbial contamination of coastal waters and reduce human health risk by bathing and consumption of seafood.

PATHOGENIC MICROORGANISMS OF RELEVANCE TO HUMAN HEALTH IN THE MARINE ENVIRONMENT

Most human pathogens are found in coastal marine environments and not in open ocean habitats. This is commonly attributed to human activities at the coast line. Therefore, we will concentrate for this study on coastal marine environments and estuaries in Europe. Bacteria are the best known marine human pathogens and *Vibrio cholera* is the best studied pathogen of marine origin. The overview about marine bacterial pathogens given in Table 1 indicates that these bacterial pathogens only belong to a very limited number of bacterial taxa, i.e. *Gammaproteobacteria*, *Epsilonproteobacteria*, *Firmicutes* and *Actinobacteria*. Some of these bacteria are well known as indigenous marine bacteria, e.g. *Vibrio* and *Aeromonas* species, whereas others are of allochtonous origin, e.g. *Salmonella* sp. and *Helicobacter pylori* (Carbone *et al.,* 2005). In addition to the well known pathogenic species or strains, “new species or strains” have to be considered as potential health threat when cases of infections start increasing. For example, infections by *Mycobacterium marinum*, *Erysipelothrix rhusiopathiae* (Oliver, 2005) or high abundance of potentially pathogenic bacteria, such as *Arcobacter* spp. (Maugeri *et al.,* 2004), have been detected recently in marine coastal waters. Furthermore, the occurrence of pathogenic bacteria equipped with new virulence or antibiotic resistance genes (by horizontal gene transfer, e.g. by bacteriophage transduction), or hybrid organisms generated from two genomes of non-pathogenic bacterial strains (Bisharat *et al.,* 2005) can result in highly virulent bacteria representing a sudden and unexpected threat to human health. Thus, we have to take into account the threat by emergence and re-emergence at the species and clonal level. This threat is a great challenge for the provision of the adequate detection tools and prevention measures.

Table 1. Pathogenic bacteria in the marine environment.

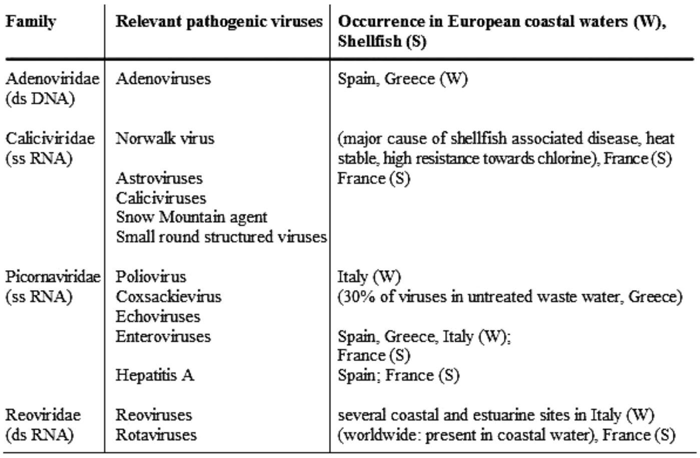


Taxonomy/species	Virulence * (estimated)	Ability to grow in the marine environment	Occurrence in European coastal water(W), shellfish (S)
Gammaproteobacteria			
Aeromonas hydrophila	low	yes/ up to high abundance	
Vibrio cholerae	high	yes	W (France, Italy, Spain)
Vibrio vulnificus	high	yes	W (Denmark, France, Italy)
Vibrio parahaemolyticus	high	yes	W (France, Italy)
Vibrio mimicus	high	yes?	
Vibrio alginolyticus	low	yes	W (Italy)
Salmonella spp.	low to high	no/?	W/S (EU)
Shigella spp.	high	no	
Escherichia coli	low to high	no/?	W/S (EU)
Plesiomonas shigelloides	low	no/?	
Epsilonproteobacteria			
Campylobacter spp.			W (Italy, Finland)
(C. lari, C. coli, C. jejuni)	low to high	no/?	S (*C. lari)* (Netherlands)
Helicobacter pylori	high	no/?	W (sewage impacted coastal water, Italy)
Arcobacter butzleri	low	yes	W (Italy, Baltic Sea)
Arcobacter skirowii	low	yes	W (Italy)
Arcobacter cryaerophilus	low	yes	W (Italy)
Firmicutis			
Clostridium perfringens	low	no/?	
Erysipelothrix rhusiopathiae	low	no/?	
Actinobacteria			
Mycobacterium avium paratub.	low	no/?	
Mycobacterium marinum	low	yes	

Legend:
* For an estimated minimum infection dose (see Pommepuy *et al.*, 2004).
? No significant data available.
W Pathogen detected in water sample.
S Pathogen detected in shellfish.

The detection of pathogenic viruses in marine environments is much more recent than that of bacteria, with the first detection of Coxsackievirus in 1947 by Dalldorf and Sickles (for a review see Griffin *et al*., 2003). The four major families of viruses found are *Adenoviridae*, *Caliciviridae*, *Picornaviridae* and *Reoviridae*, as indicated in Table 2. These families comprise double stranded (ds) DNA, single stranded (ss) RNA and ds RNA viruses. All known pathogenic viruses that pose a significant health threat in the marine environment are transmitted via the fecal-oral route and are therefore called "enteric viruses".

Table 2. Pathogenic "enteric viruses" (transmitted from feces) in coastal environments (data from Griffin *et al.,* 2003).



Family	Relevant pathogenic viruses	Occurrence in European coastal waters (W), Shellfish (S)
Adenoviridae (ds DNA)	Adenoviruses	Spain, Greece (W)
Caliciviridae (ss RNA)	Norwalk virus	(major cause of shellfish associated disease, heat stable, high resistance towards chlorine), France (S)
	Astroviruses	France (S)
	Caliciviruses	
	Snow Mountain agent	
	Small round structured viruses	
Picornaviridae (ss RNA)	Poliovirus	Italy (W)
	Coxsackievirus	(30% of viruses in untreated waste water, Greece)
	Echoviruses	
	Enteroviruses	Spain, Greece, Italy (W); France (S)
	Hepatitis A	Spain; France (S)
Reoviridae (ds RNA)	Reoviruses Rotaviruses	several coastal and estuarine sites in Italy (W) (worldwide: present in coastal water), France (S)

In addition to viruses, three genera of zoonotic protozoa, i.e. *Giardia*, *Cryptosporidium* and *Toxoplasma*, are detected in marine environments (Fayer *et al*., 2004) including coastal water, sediments and shellfish. The origin of the protozoa are feces of humans or (mostly domestic) animals. Protozoa survive well as cysts that are small, buoyant, and resistant to most environmental influences, even to desinfectant, and remain infective in moist environments for long periods (up to a year); thus protozan cysts are by far more resistant to environmental stress than viruses and bacteria (Nasser *et al*., 2003). The occurrence of land derived protozoan species like *C. parvum* in shellfish indicates their land origin. Infection by these protozoa in the marine environment is observed for a large set of marine mammals.

The pathogens found in the marine environment are responsible for a broad spectrum of acute and chronic human diseases, e.g. gastroenteritis, ocular and respiratory infections, hepatitis, myocarditis, meningitis, and neural paralysis. Most marine pathogens find their origin in feces, with only a very few “autochthonous bacterial pathogens” (e.g. vibrios) able to grow in the marine coastal environment.

MECHANISMS INFLUENCING SURVIVAL OF PATHOGENS IN THE MARINE ENVIRONMENT

The survival of human pathogens in the marine environment is controlled by several key factors, such as grazing, solar radiation and temperature, organic matter availability, and particle load. All these factors are greatly influenced by man-made eutrophication and climate change.

MECHANISMS REDUCING SURVIVAL OF PATHOGENS IN THE MARINE ENVIRONMENT

Grazing: all pathogenic microorganisms are submitted to grazing in the marine environment to a certain extent. Most relevant grazers are flagellates, ciliates, zooplankton (cladocerans) and filter feeding bivalves, ascidians, polychaetes and sponges. Grazing implies ingestion and digestion of the microorganisms. Bacteria are to some extent eliminated by grazing. Viruses are the least affected by grazing due to their small size. On and in filter feeding bivalves a strong enrichment of pathogenic bacteria and viruses is usually observed (Miossec *et al*., 2000; Muniain-Mujika *et al*., 2003). In general, bivalves show an intense enrichment for heterotrophic bacteria, especially for vibrios (Stabili *et al*., 2004) compared to the surrounding coastal water, caused by the concentration by filter-feeding plus growth in the high organic nutrient environment of the bivalves. While most gram-negative marine bacteria are considered to be well digested by bivalves (Birkbeck and McHenery, 1982), this is not the case for many pathogenic bacteria. Especially for vibrios, defense mechanisms against digestion and humoral response of the bivalve have been demonstrated that allow survival and even growth within the bivalve gut (Charles *et al.,* 1992; Pruzzo *et al.,* 2005a).

Solar radiation: UV radiation is able to destroy viruses and bacteria. Since UV radiation decreases with water depth and turbidity, bacteria and viruses in deeper water layers and/or particle rich water are less stressed by UV radiation than in clear surface water.

Temperature: water temperature has a different effect on bacteria and viruses. Most pathogenic bacteria are more abundant at higher water temperature, because survival or even growth increases at higher temperature; this is especially valid for the autochthonous bacteria (e.g. vibrios). With decreasing water temperature, an increased fraction turns into a viable but non-culturable (VBNC) state (Huq *et al.,* 2000), the infectivity and the survival rate decreases. By contrast, some enterobacteria survive longer at colder temperatures (Pommepuy *et al*., 2004). Viruses survive longer and display a higher rate of infectivity at lower temperature. Protozoa survive for a long time (as cysts up to a year or even longer) regardless of the temperature (Fayer *et al.,* 2004).

Bacteriophages: bacteriophages can infect bacteria and lead (later on - and this time period is highly variable) to lysis and thus elimination of the bacterial cells. Bacteria in the marine environment can either be infected before they enter the marine environment (e.g. in the intestine or the sewage treatment facility) or be infected in the marine environment. Due to their specificity, the host spectrum of a specific phage is very restricted, usually to a specific bacterial species. Since the probability to be infected by a virus of the right specificity is dependent on the abundance/density of a bacterial host, bacteria with a high abundance in the coastal environment - such as vibrios or *Aeromonas* sp. - are much more likely to be infected and killed by viruses in the marine environment (Jiang *et al*., 2003). For bacteria unable to grow in the marine environment, infection by viruses of marine origin is not likely; by contrast the presence of bacteriophages specific for enterobacteria can be used as a tracer for fecal contamination in shellfish or in coastal water (Dore *et al.,* 2000).

MECHANISMS SUPPORTING SURVIVAL OF PATHOGENS IN THE MARINE ENVIRONMENT

Particles: in general, attachment of microorganisms to particles improves their survival and infectivity (Brettar and Höfle, 1992; Griffin *et al.,* 2003; Maugeri *et al.,* 2004). For bacteria, enhanced nutrient conditions, shelter from grazing and UV irradiation are the main features provided by particles to improved survival or growth conditions. For viruses, shelter from UV irradiation supports survival and infectivity. For protozoa, particle attachment seems irrelevant for survival in the water. For all microorganisms, particles may serve as a vehicle for transfer to the sediment.

Attachment to plankton: is of special relevance for the survival and growth of pathogenic bacteria in the marine environment. Attachment can occur to zooplankton and phytoplankton that both provide increased availability of organic nutrients and shelter from UV and grazing. For vibrios, attachment to zooplankton is of high relevance for survival, growth and distribution (via transport by the animal host and/or its feces) (Baffone *et al.,* 2006; Maugeri *et al.,* 2004).

Attachment to bivalves: provides for some pathogenic marine bacteria (e.g. vibrios) similar conditions like attachment to plankton, concerning the high nutrient environment with protection from grazing and UV, allowing improved survival and growth compared to the surrounding water (Cavallo and Stabili, 2002).

Sediment: all pathogenic microorganisms show increased concentrations, prolonged survival and infectivity in sediments, especially at its surface (Fayer *et al.,* 2004; Griffin *et al.,* 2003; Brettar and Höfle, 1992). Reduced grazing and protection from UV irradiation could be important for the improved survival conditions.

Organic nutrients: organic nutrients improve the survival or growth conditions of bacteria in coastal waters. They can derive from autochthonous algal growth or land derived organic matter (river discharge, sewage, run off). Highest concentrations of organic nutrients are found on the surface of organisms like algae or copepods. Attachment to these surfaces may therefore provide a favourable high nutrient environment for bacteria, that is also used by pathogenic bacteria, especially by members of the genus *Vibrio*.

INFLUENCE OF EUTROPHICATION AND GLOBAL WARMING

Man-made eutrophication: the eutrophication of coastal waters due to the addition of inorganic nutrients increases the production of algal biomass. Therefore, eutrophication improves the survival of pathogenic bacteria by increasing the availability of organic nutrients and particles; the increasing particle load will also improve the survival of pathogenic viruses.

Global warming raises the temperature of coastal water, that is expected to improve the survival conditions for pathogenic bacteria. For viruses, the higher temperatures could be expected to decrease the survival. However, the effect of eutrophication may in many coastal environments counteract the temperature effect on viruses. Additional effects of global warming are increased frequency of heavy rainfalls, storms and floods. These heavy rain periods lead to severe problems with run off, sewage transport and treatment and cause increased fecal contamination of coastal ecosystems.

EMERGING THREATS AND POTENTIAL MECHANISMS TO INCREASE PATHOGENICITY OF MICROORGANISMS

Highly pathogenic microorganisms can either be directly introduced into the marine environment, or increase their pathogenicity in the marine environment. Major sources for introduction are sewage, with sewage derived from hospitals, and inadequately treated sewage especially from large urban areas representing a great threat. Additionally, ballast water has to be taken into account - not only for toxic algal blooms but also for the transfer of highly pathogenic microorganisms (Peperzak, 2005).

Mechanisms for increasing the pathogenicity for bacteria are i) horizontal gene transfer that allows acquisition of genes responsible for virulence or antibiotic resistance from other (usually related) bacteria or via bacteriophages, or - as recently shown - ii) formation of a hybrid highly pathogenic organism generated from two genomes of non-pathogenic bacterial strains (Bisharat *et al*., 2005). Horizontal gene transfer is more likely with increasing density of a pathogenic specific bacterial species or genus, e.g. more likely for vibrios than for enterobacteria and for sites of high concentration, such as gills of bivalves (Miller, 2001). Bacteriophages can serve as vehicles for nucleic acids of bacterial origin (genomic or plasmid DNA) that can lead to transduction of bacteria. An eminent example is the cholera toxin which is encoded by a lysogenic bacteriophage (Faruque *et al.,* 1998).

DETECTION TECHNOLOGIES FOR MICROBIAL PATHOGENS IN SEAWATER

Currently, hygienic quality and potential health risk from the exposure to seawater and the consumption of contaminated seafood is assessed by the cultivation of indicator bacteria. This classical microbiological methodology relies on the cultivation of specific bacteria, e.g. plate counts (CFU) of coliforms or plaque forming units (PFU), and has a variety of serious drawbacks, like no correlation to many microbial pathogens and no valid identification of the pathogen. Therefore, this methodology is completely inappropriate for the detection of pathogens in seawater. For this reason state-of-the-art molecular detection methodology has to be considered. Advanced methodology for environmental detection of microorganisms depends on: i) the type of microorganism, ii) the level of taxonomic resolution to be achieved, iii) the detection limit to be reached and iv) the number of samples to be analysed per Euro. The universal approach currently pursued is to analyse nucleic acids, extracted directly out of seawater, with a suite of molecular methods ranging from PCR and DNA array-based techniques to immuno-capturing and fluorescence *in situ* hybridisation (FISH). Using quantitative PCR techniques, such as real-time PCR, enabled during the last years to detect and quantify the pathogenic viruses and bacteria in seawater without the need of growing them in cell cultures (Fey *et al.,* 2004; González-Escalona *et al*., 2006). Although protozoa, like *Cryptosporidium*, can now be precisely quantified using immunofluorescence detection and/or immunofluorescence microscopy, species specific identifications asks for PCR-based detection because *Cryptosporidium* species cannot be differentiated by immunological methods (Bialek *et al*., 2002). All these advanced molecular detection methods require a high degree of expertise and sophisticated technology and are far from being routine use for seawater analysis. Therefore, there is a great need for technological improvement, standardisation, validation and automation of the molecular detection technology. Validation of molecular detection methods for the environmental detection of microorganisms is a very recent field, highly recommended for quality control of drinking water by a recent OECD report (OECD, 2003).

Molecular techniques can also be used for the detection of fecal contaminations in general and not only for the detection of a specific pathogen. This analysis, called microbial source tracking (MST), is highly relevant for the assessment of human health risks and for the identification of the route of infection and its prevention. Currently several approaches are pursued: 1) speciation – finding microbial species indicative of the source, 2) biochemical marker substances such as fecal sterols, 3) assemblages and ratios – comparison of two or more fecal bacteria or viruses population ratios, and 4) DNA fingerprints using environmental DNA and specific phylogenetic genes of fecal bacteria (Simpson *et al*., 2002). The area of MST is a rapidly developing field in environmental and food microbiology and will allow to discriminate between fecal contaminations from human or animal sources (Blanch *et al*., 2006).

CURRENT MONITORING SCHEMES FOR COASTAL MARINE ENVIRONMENTS AND SEAFOOD IN EUROPE

The current monitoring of the hygienic condition of coastal bathing water is regulated in the EU by the Directive 76/160/EEC modified in 2004 based on the WHO guidelines (WHO, 2003). The Directive provides threshold values for culture based detection (CFU) of fecal enterococci (*Enterococcus faecalis* / *faecium*) and *Eschericha coli*. It does not provide any detection of viruses and non-culturable bacteria and is based on these indicator data alone. An assessment of the health risk and the protection values of the current Directive thresholds is under way (EU project EPIBATHE http://www.aber.ac.uk/iges/research/epibathe/).

MONITORING OF THE HYGIENIC QUALITY OF COMMERCIAL SEAFOOD

The correct evaluation of the bacteriological quality of water and, therefore, of cultured shellfish, requires (i) the utilization of culture dependent and independent assays to properly evaluate the presence of faecal indicators and potentially pathogenic environmental bacteria (in culturable and non culturable state) both in ambient seawater and in bivalves, collected before, during and after depuration; (ii) the analysis of the state of activity and pathogenicity of bacteria under diverse environmental conditions, and (iii) the analysis of environmental biotic and abiotic factors that may influence survival, abundance and pathogenicity of bacteria and viruses.

SOURCES AND TRANSMISSION MECHANISM OF MICROBIAL CONTAMINATION (see Figures 1-3)

Sewage: well-treated sewage is intended to contain low numbers of bacteria, viruses and protozoa; the load of pathogenic microorganisms increases with decreasing treatment procedure/quality. Major problems are heavy rain periods, storm events or floods that flush untreated or not efficiently treated sewage into rivers and coastal water. In all industrialized countries, pathogenic microorganisms including bacteria, viruses and protozoa can be found in coastal waters, even in regions with high quality sewage treatment procedures. The load of pathogenic organisms in coastal water and shellfish increases during heavy rain periods, as well as the incidence of disease (Ahn *et al.,* 2005; Miossec *et al.*, 2000). Additionally, also treated and disinfected wastewater still may have a high load of pathogenic organisms that ranges on average

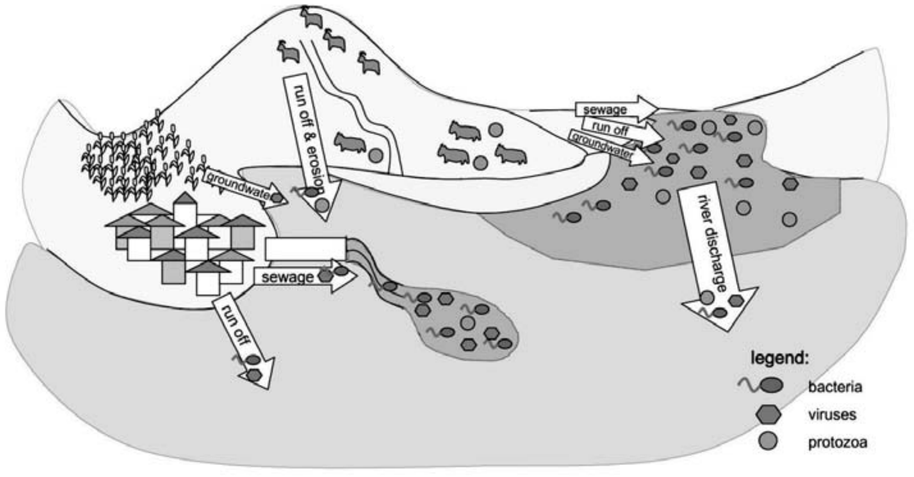


Fig. 1. Transfer of pathogenic microorganisms from land to sea.
Main transfer routes of pathogenic microorganisms to the sea are sewage, run off and erosion, groundwater and river discharge (see arrows). Major sources of pathogenic bacteria are feces of human and (mostly domestic) animals. The importance of the different transfer routes varies with degree of urbanization, sewage transport and treatment, agricultural activities, cattle density, landscape and vegetation. In addition to these routes, ship traffic, ballast water, and bathing activities may contribute to microbial contamination of coastal water. Some pathogenic bacteria (e.g. vibrios) are considered as autochthonous marine microorganisms that live and grow in coastal sea water.

between 30 and 80% (the lower values for bacterial pathogens, the higher for protozoan cysts, and the viruses in between) compared to concentrations in untreated sewage as shown in a recent 1-year-monitoring of sewage treatment plants in Florida (Harwood *et al.,* 2005).

Sewage sludge: disposal of sewage sludge in river and coastal regions can be a major source of viruses and protozoa, either if disposal is done directly into the aquatic ecosystem, or after flushing during heavy rain events.

Run off/ floods: run off will carry pathogenic microorganisms from soil/street surface and - with respect to the strength of rain falls - overloaded sewers, rain water collectors and sewage treatment facilities to rivers and coastal water.

Groundwater: microbiologically contaminated groundwater may seepage directly into coastal water or be transferred via river water. For viruses high transfer rates from groundwater aquifers, especially in limestone, to costal water have been observed. Major contaminators of groundwater are households (with private sewers), livestock, and leaking sewage pipes.

River discharge: rivers often carry high loads of pathogenic microorganisms due to high impact by run off, sewage and groundwater. Loads are most pronounced after periods of heavy rains. Therefore, in areas affected by the river plume, increased coastal water contamination has to be taken into account.

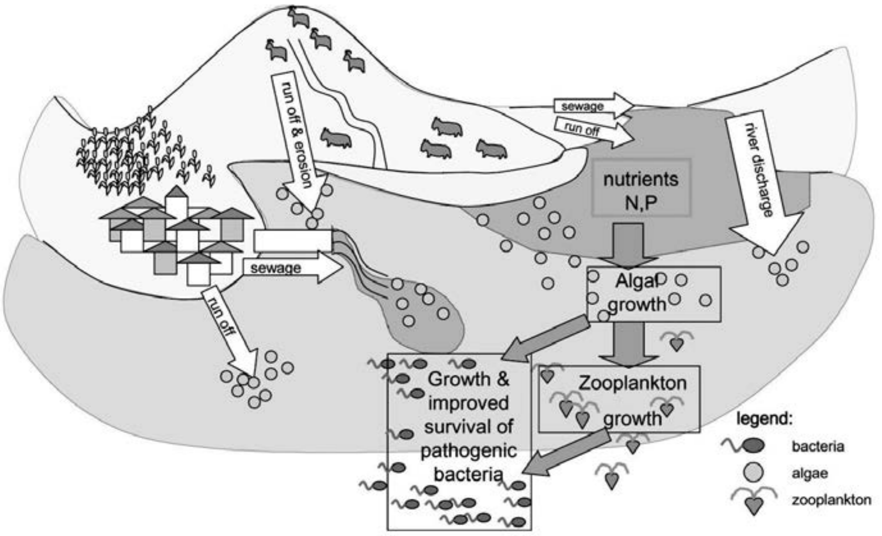


Fig. 2. Impact of eutrophication on pathogenic microorganisms.
The same transfer routes that carry pathogenic microorganisms also transport nutrients (nitrogen (N), phosphorus (P)) to the sea, thus causing eutrophication (white arrows indicate nutrient transfer). Eutrophication supports growth of algae and - as a response to algal growth - growth of zooplankton. The high abundance of phyto- and zooplankton supports the growth and survival of the autochthonous pathogenic bacteria (e.g. vibrios). Also, land-derived bacteria and viruses profit from eutrophication, due to increased amounts of particulate matter that protect from UV, and provide sites for attachment and transport.

In summary, feces of men and domestic animals are the major sources of pathogenic microorganisms. Paths from feces to the final disinfection, i.e. the sewage treatment plant, are difficult to control. Especially, for periods of heavy rains and floods it does not seem to be achievable. Further, viruses and protozoa are usually only reduced but not eliminated in sewage treatment plants (Harwood *et al*., 2005); those deposited in the sewage sludge are another source of contamination unless effective further treatment is achieved.

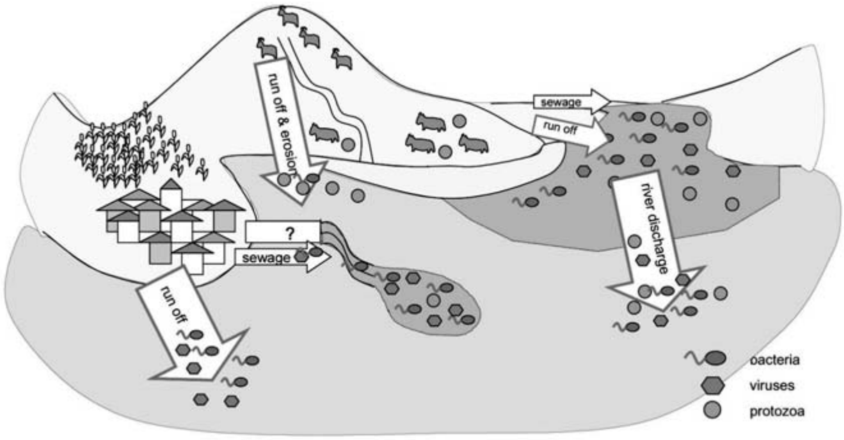


Fig. 3. Impact of heavy rain fall on transfer of pathogenic microorganisms.
During periods of heavy rain, run off and rivers transfer increase amounts of pathogenic bacteria to coastal waters. Major sources of contamination in urban run off are human and animal feces derived from overflowing or leaking sewer lines, septic tanks, and impervious surfaces (e.g. asphalt). In heavy rain periods, sewage partially does not reach the sewage treatment plant (especially valid for mixed sewer systems - rainwater/sewage), the high water load will counteract a proper sewage treatment and a large fraction of the pathogenic microorganisms will be transported to the recipient ecosystem, e.g. river or coast. In rural area, feces from cattle can contribute to contamination - either when flushed from field (manure) or pasture or directly from the storage site (deliberate or accidental). While urban run off will have a high load of bacteria and viruses, for run off from sites with cattle or manure application, additionally protozoa are of high relevance.

CONSIDERATIONS FOR INTERRUPTING THE FECAL-ORAL INFECTION ROUTE AND STEPS TOWARDS AN INTEGRATED COASTAL MANAGEMENT SCHEME

A reduction of the transmission zoonotic protozoa from pastures to river and coastal water can be most effectively achieved by “landscape solutions” (see Figure 4), i.e. riparian buffer strips, measures that reduce erosion and rapid run off, such as reforestration, and renaturation of rivers and wetlands that improves the “filtering capacity” of the landscape. These measures will counteract in the same way non-point nutrient sources, i.e. transfer of nutrients (especially nitrogen and phosphorus compounds) from the landscape to the water and thus reduce eutrophication of coastal waters.

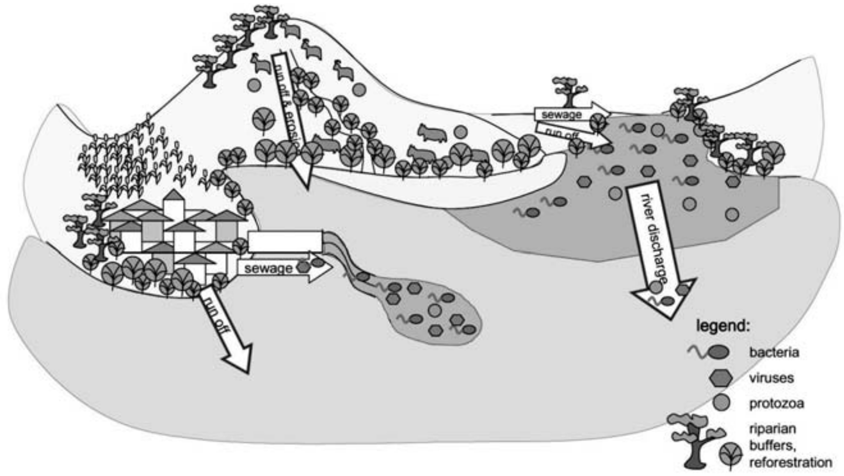


Fig. 4. Reduction of the load of pathogens due to run off by “landscape approaches”.
In urban and in rural areas “landscape approaches” are able to reduce the amount of run off and the transfer of pathogenic microorganisms during periods of heavy rain. Suitable landscape approaches are the restoration and protection of riparian buffer strips, reforestration, restoration of areas with degraded soil and gully erosion, restoration of wetlands, and - especially in settlements - the reduction of impervious surfaces (asphalt, pavement, concrete etc.). These measures enhance the “filtering capacity” and increase the retention capacity for water in the landscape. Even in cities, a consequent reduction of impervious surfaces or an increase of the “green surface” (e.g. vegetated roofs, gardens instead of parking lots) will reduce the amount of stormwater and thus the load of microorganisms transported by run off. These “landscape approaches” will concomitantly reduce the heavy load of nutrients transported during periods of heavy rain to surface water, thus counteract eutrophication.

Transfer or storage of pathogenic organisms in sewage water cannot be done, it seems, without occasional high contamination of surface, ground water and coastal water. A solution to the risk of storage and transport would be a rapid on site extinction of the pathogenic bacteria and viruses (see Figure 5). Efficient procedures to reduce the load of pathogens (bacteria, viruses, protozoa, helminths) in human and animal waste in a short period of time (one to a few days) are those that include a step with heating of the waste, such as composting (>60°C, due to microbial heating) and the thermophilic production of biogas by methanogenesis (> 50°C). Critical to these procedures is that they reach high temperatures (above 50°C) for the required period of time (one to a few days, dependent on the nature of the pathogens present and the final temperature of the applied procedure). These hygienic measures for waste treatment are technologically well established and could contribute to a healthy and sustainable life on the European coastline and are also helpful when using biogas production (Verstraete *et al.,* 2002; Erikson *et al.*, 2004).

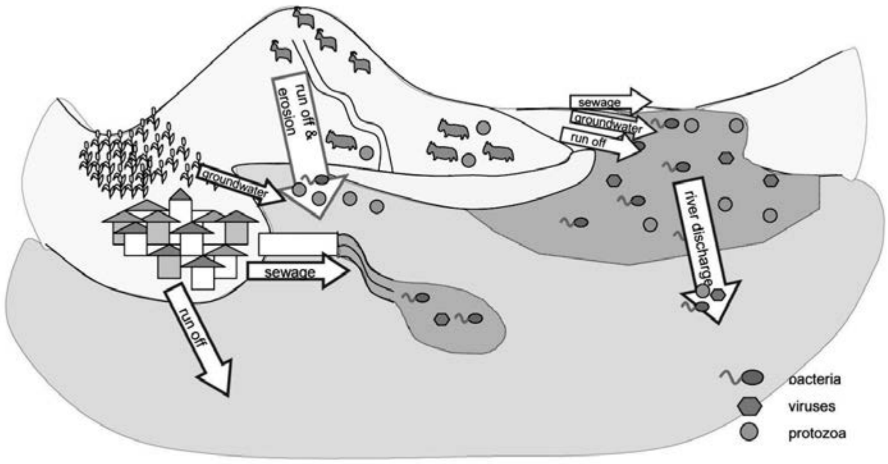


Fig. 5. Reduction of transfer of pathogenic microorganisms by “on site sanitation”.
On site sanitation means treatment of human and animal feces without long distance transfer directly after production by heat producing procedures like composting or thermophilic biogas production. These heat producing procedures rapidly reduce the number of pathogenic viruses, protozoa and bacteria. Avoidance or reduction of transport of feces-containing material reduces the risk of loss and leakage during the transport. Cattle manure can be efficiently used for biogas production - with concomitant elimination of the pathogenic microorganisms. Animal or human feces freed from pathogenic microorganisms are safe to be used as manure in agriculture without risk for field workers and consumers, or contamination of water bodies, especially in periods of heavy rains. Thus, on site sanitation would reduce the risk of contamination of groundwater, surface and coastal water and could essentially improve public health and contribute to a sustainable management of the coastal zone.

A reduction of autochthonous pathogenic bacteria (of special relevance vibrios) could be supported by measures to reduce coastal eutrophication. Eutrophication increases growth of phytoplankton - the major deliverer of organic matter that supports the growth of the autochthonous pathogenic bacteria (Mourino-Perez *et al*., 2003). Additionally, phytoplankton is fed upon by zooplankton - another major site for vibrio growth and dispersal. To reduce coastal eutrophication point sources (sewage discharge) and non-point sources (see “landscape solutions” above) of nitrogen and phosphorus have to be controlled.

In conclusion, the hygienic treatment of waste and waste water together with measures to reduce transfer of pathogens and nutrients from point and non-point sources could provide the basis for an integrated coastal management scheme that could greatly reduce the occurrence and growth of pathogens in coastal environments and ensure safe bathing and seafood consumption.

General conclusions and future research needs

There is a rather limited knowledge on the microbiological principles governing the prevalence and pathogenesis of human microbial pathogens in the marine environment. The reasons for this lack of knowledge are manifold: i) precise detection, identification and quantification of microorganisms in water is difficult and only possible with a combination of classical and molecular methods; ii) the virulence of waterborne pathogens varies greatly depending on environmental conditions and the type of pathogen; and iii) the transmission of waterborne infections to humans via seawater is a complex process depending on the type of pathogen, infectious dose, immune status of the human and a multitude of other factors.

We foresee the following future research needs and propose a research strategy (Figure 6):

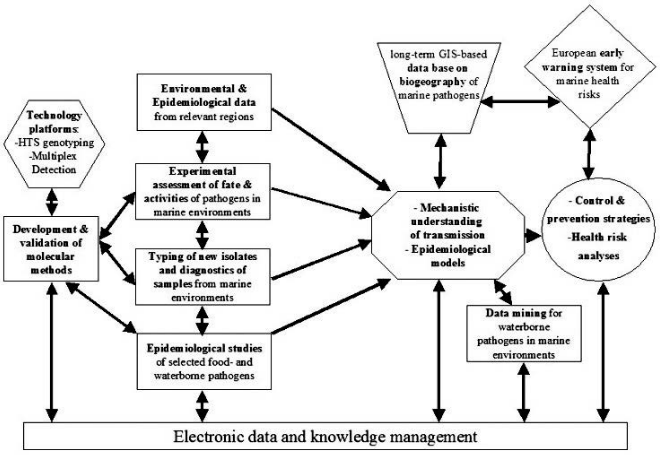


Fig. 6. Overall strategy to assess transmission of marine pathogens to humans and improve public health in Europe.

- development, validation and standardization of rapid detection methods for the most relevant pathogenic viruses and bacteria in seawater and seafood;
- abundance studies of major pathogens in the marine environment to elucidate their biogeography and their environmental control factors;
- thorough investigation of emerging marine pathogens including genomics, ecophysiology and host-pathogen-interactions;
- studies on survival of pathogenic microorganisms, including emerging ones, in marine environments under all relevant conditions able to influence survival (e.g. temperature, UV, water, particle attached, sediment, etc.);
- studies on sanitation and sewage treatment conditions with respect to elimination of pathogenic bacteria, viruses and protozoa;
- study of the epidemiology of seawater-borne and seafood-borne diseases originating from human pathogens of the marine environment to improve public health.

Viabilité et maintien de la virulence des bactéries pathogènes dans l’environnement marin

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VIABILITÉ ET CULTIVABILITÉ

L’étude des caractéristiques phénotypiques et génétiques des bactéries est basée sur la culture bactérienne sur milieu nutritif. Cette « obligation » de cultiver les bactéries pour pouvoir les détecter et les identifier a défini la notion de bactéries détectables par des techniques de cultures et de bactéries capables de se multiplier sur un milieu nutritif adapté. Cette « cultivabilité » des bactéries a été associée à la viabilité. Cultivabilité et viabilité sont ainsi devenues des synonymes généralement admis dans la littérature (Postgate, 1969 ; Hattori, 1988 ; Kell *et al*., 1998). La cultivabilité seule pouvant refléter la viabilité, il pouvait paraître évident que la non-cultivabilité reflétait la non-viabilité, c’est-à-dire qu’une bactérie non cultivable était une bactérie « morte ». La non-cultivabilité a été ainsi associée à la non-viabilité donc à la mortalité.

Le principe d’équivalence viabilité-cultivabilité a servi à établir un résultat fondamental dans la gestion des eaux en santé publique : celui du déclin progressif des populations entériques dans l’environnement aquatique naturel. Les bactéries entériques étant en premier lieu adaptées aux conditions régnant dans le tube digestif de leur hôte, elles sont allochtones des milieux aquatiques. Ne pouvant faire face durablement aux conditions adverses qu’elles y rencontrent, telles l’oligotrophie, le rayonnement solaire (visible et proche UV), la salinité, la température, la prédation et la compétition du biota autochtone, elles ne s’y maintiennent pas (Rozen et Belkin, 2001 ; McFeters et Singh, 1991). En pratique, leur perte de viabilité est révélée par leur perte de cultivabilité.

La mise en évidence du maintien de la viabilité cellulaire par le test du « Direct Viable Count » (DVC) (Kogure *et al*., 1979) des cellules bactériennes non-cultivables a remis en cause définitivement le concept de la « mort » des bactéries non-cultivables. L’utilisation des techniques de microscopie en épifluorescence pour dénombrer directement les cellules bactériennes (fluorochromes, anti-corps fluorescents, DVC) a montré que des cellules de bactéries d’intérêt sanitaire comme *Escherichia coli*, *Vibrio cholerae*, *Samonella enteritidis*, étaient présentes dans les milieux aquatiques alors qu’elles n’étaient pas « cultivables » sur milieux de culture (Xu *et al*., 1982 ; Roszak *et al*., 1984). De telles cellules « viables mais non cultivables » (VNC), « viable-but-non-culturable » (VBNC) de bactéries d’intérêt sanitaire pourraient alors poser un problème de santé publique de par leur présence dans un échantillon alors que les méthodologies microbiologiques traditionnelles de contrôle ne permettent pas de les détecter et de les dénombrer (Roszak *et al*., 1984).

RÉALITÉ DES BACTÉRIES « VIABLES MAIS NON CULTIVABLES »

Lorsqu'une population bactérienne est soumise à l'action d'un ou plusieurs facteurs limitants (le rayonnement solaire, la salinité, l'absence de nutriments dans le cas des milieux aquatiques, par exemple) le premier effet visible de cette action est la diminution du nombre de bactéries recensées au moyen d'un milieu de culture, donc du nombre de bactéries cultivables. La mort cellulaire ne peut être affirmée que dans le seul cas où la cellule n'est plus visible après marquage par un fluorochrome spécifique des acides nucléiques (Roszak et Colwell, 1987b).

Le phénomène de la perte de la cultivabilité apparaît chez toutes les bactéries normalement cultivables dès lors qu'elles sont soumises à un facteur stressant (Roszak et Colwell, 1987a), notamment chez toutes les espèces bactériennes allochtones aux écosystèmes aquatiques comme les pathogènes d'origine entérique (Barcina *et al*., 1997). La perte de la cultivabilité des bactéries d'intérêt sanitaire pourrait conduire à sous estimer leur présence par les méthodes traditionnelles de contrôle qui utilisent les milieux de culture pour évaluer la qualité sanitaire des eaux (Grimes *et al*., 1986 ; Mc Feters et Singh, 1991 ; Monfort et Baleux, 1991). La perte de la cultivabilité des bactéries pathogènes pose le problème du maintien ou non du pouvoir pathogène de ces cellules devenues VNC (Colwell *et al*., 1988 ; Rahman *et al*., 1996 ; Baleux *et al*., 1998).

Cet état viable mais non cultivable a été étudié sur différentes bactéries pathogènes, notamment sans être exhaustif *Aeromonas salmonicida* (Allen-Austin *et al*., 1984), *Campylobacter jejuni* (Jones *et al*., 1991 ; Rollins et Colwell, 1986), *Escherichia coli* entérotoxinogène (Singh *et al*., 1986), *Klebsiella pneumoniae* (Yu et McFeters, 1994), *Salmonella enteritidis* (Roszak *et al*., 1984), *Salmonella paratyphi*, *Staphylococcus* (Cornax *et al*., 1990), *Salmonella typhimurium* (Baleux *et al*., 1998 ; Caro *et al*., 1999), *Vibrio cholerae* O1 (Colwell *et al*., 1985), *Vibrio vulnificus* (Oliver, 1995), *Yersinia enterocolitica* (Singh *et al*., 1985).

PHYSIOLOGIE DES CELLULES VIABLES MAIS NON CULTIVABLES

Afin de déterminer si une cellule bactérienne non cultivable est cependant viable, il est nécessaire de rechercher si elle présente encore une activité physiologique. Par suite du développement des méthodes cytométriques appliquées à l'analyse détaillée de la cellule bactérienne, il est possible de décrire certaines fonctions physiologiques (Monfort *et al*., 1995). En utilisant ces marqueurs et au moyen des techniques cytométriques, la mesure des paramètres morphométriques et du contenu cellulaire en acides nucléiques (fluorescence de la sonde fixée sur les acides nucléiques) a permis de montrer que sous l'action de facteurs environnementaux comme la salinité et la privation nutritive, les cellules non cultivables de *Salmonella salamae* et *typhimurium* présentent une réduction de leur taille et de leur contenu en ADN (intégrité génomique) (Lebaron et Joux, 1994 ; Monfort et Baleux, 1994 ; Baleux et Got, 1996 ; Baleux *et al*., 1998 ; Caro *et al*., 1999).

La mise en évidence d'une activité physiologique et métabolique au moyen de ces différentes sondes et tests permet de dire que ces cellules présentent une « viabilité », et donc de confirmer qu'elles sont viables mais non cultivables (VNC), « viable-but-nonculturable » (VBNC) (Kell *et al*., 1998). En utilisant ces techniques cytométriques, différents travaux ont mis en évidence que des cellules bactériennes d'intérêt sanitaire soumises à des conditions environnementales stressantes dans les milieux aquatiques, notamment chez des bactéries comme *Escherichia coli*, *Salmonella typhimurium*, *Vibrio vulnificus*, deviennent des cellules viables mais non cultivables (Firth *et al*., 1994 ; Lopez-Amoros *et al*., 1995 ; Porter *et al*., 1995 ; Joux *et al*., 1997 ; Baleux *et al*., 1998 ; Caro *et al*., 1999 ; Muela *et al*., 1999). La plupart des travaux montre en fait plusieurs niveaux de viabilité cellulaire en fonction de l'exposition aux facteurs limitants. Une hiérarchisation dans l'évolution de la physiologie cellulaire est même concevable. C'est ainsi que les cellules devenues non cultivables perdent, en premier, l'activité de synthèse protéique, ensuite l'activité respiratoire, leur intégrité membranaire, et enfin l'intégrité du génome (Joux *et al*., 1997 ; Baleux *et al*., 1998 ; Caro *et al*., 1999).

MAINTIEN DU POUVOIR PATHOGÈNE DES CELLULES VIABLES MAIS NON CULTIVABLES PATHOGÈNES

Le maintien de la viabilité de cellules bactériennes pathogènes VNC pose le problème du maintien concomitant de leur virulence. En effet il reste à démontrer que la perte de la capacité de cultiver des cellules VNC s'accompagne de la perte de leur pouvoir pathogène. Colwell *et al.* (1985) ont fait la démonstration, pour la première fois, du maintien de la pathogénie de cellules non cultivables (*Vibrio cholerae* O1, *E. coli* entérotoxinogène H10407) mis en évidence par le test de l'anse intestinale ligaturée de lapin.

Des cellules de *Shigella dysenteriae* type 1 devenues VNC après un séjour dans de l'eau MilliQ conservent la présence du gène *stx*, de l'activité biologique de la toxine Shiga (ShT) et la capacité d'adhérer sur une lignée cellulaire en culture (Henle 407) ; ces cellules VNC conservent ainsi plusieurs facteurs de pathogénicité et se maintiennent potentiellement pathogènes (Rahman *et al.*, 1996). Après un séjour de sept jours dans de l'eau de mer à 5°C, des cellules de *Vibrio vulnificus* rendues VNC conservent leur pouvoir pathogène vis-à-vis du modèle souris (Oliver et Bockian, 1995). Des cellules d'une souche d'*Escherichia coli* entéropathogène devenues VNC après un séjour dans de l'eau de mer et exposition aux rayonnements solaires présentent toujours leur capacité à produire une entérotoxine mise en évidence par le test de l'anse intestinale ligaturée de lapin ou par ELISA (Pommepuy *et al.*, 1996). Colwell *et al.* (1996) ont montré qu'une souche de *Vibrio cholerae* O1 (souche vaccinale atténuée) devenue VNC après un séjour dans du PBS à 4°C provoquait des réactions diarrhéiques chez des volontaires humains.

La mise en évidence du maintien du pouvoir pathogène chez des souches bactériennes pathogènes devenues VNC n'est cependant pas systématique. Ainsi une souche de *Vibrio vulnificus* devenue VNC et maintenue pendant 32 jours dans de l'eau de mer à 5°C se révéla non pathogène vis-à-vis de l'animal test (souris) (Linder et Oliver, 1989) alors qu'avec un séjour plus court de 7 jours elles conservaient leur pouvoir pathogène (Oliver et Bockian, 1995). L'injection de cellules de *Salmonella typhimurium* devenues VNC suite à l'exposition de facteurs stressants expérimentaux à des lots de souris (voie intrapéritonéale) n'entraîne aucun état pathologique ni mortalité (Baleux *et al.*, 1998 ; Caro *et al.*, 1999). Cependant, cette même souche devenue VNC suite à une exposition à de l'eau de rivière et au rayonnement solaire provoque la mort de 20 % des souris après leur injection intra-péritonéale (Caro, 1998).

Ces quelques travaux portant sur le maintien du pouvoir pathogène chez les bactéries pathogènes VNC montrent qu'il est possible que celui-ci persiste alors que le pouvoir de cultiver est perdu. Cependant le maintien ou non de la virulence paraît être sous la dépendance de la nature du facteur stressant (rayonnement solaire, UV, salinité, température, etc.) et de la dose reçue (intensité du facteur, temps d'exposition). Compte tenu de ces résultats, il n'est pas irrationnel de dire que les cellules VNC pathogène pourraient conserver leur virulence et pourraient poser ainsi un problème en santé publique dans les milieux marins-côtiers.

REVIVISENCE OU RECULTIVABILITÉ

Aujourd'hui l'état viable mais non cultivable des cellules bactériennes est reconnu par la majorité des microbiologistes (Kell *et al.*, 1998 ; Dixon, 1998), et plus particulièrement par ceux du domaine de l'écologie bactérienne comme l'ont montré les nombreux travaux cités dans le présent article. Par contre le retour à la cultivabilité des cellules VNC, tel que montré dans les travaux publiés, semble plus controversé (Bogosian *et al.*, 1998 ; Bogosian *et al.*, 2000 ; Bogosian et Bourneuf, 2001). Il est important de reconnaître à une cellule bactérienne, bien définie comme non cultivable, la capacité nouvelle de réactiver son pouvoir de cultiver désigné par le terme « resuscitation » en langue anglaise ou « reviviscence » en français.

Opérationnellement, l'étude de la reviviscence des cellules VNC est souvent entreprise sur une population de cellules non cultivables. L'apparition de cellules cultivables dans cette population permet alors de conclure à l'aptitude des cellules VNC à la reviviscence. Cette conclusion n'est possible qu'à la condition de pouvoir exclure tout phénomène de multiplication de quelques rares cellules qui seraient restées cultivables mais non détectables car en nombre trop faible, et de ce fait inférieur à la limite de détection de la méthode utilisée (Oliver, 1995 ; Whitesides et Oliver, 1997). Aussi, il est important de reconnaître qu'il existe une probabilité que les populations non cultivables testées lors des expériences de reviviscence contiennent en fait quelques cellules cultivables, cette probabilité devant être estimée et prise en compte dans l'analyse des résultats.

Plusieurs études ont montré l'aptitude à la reviviscence et la virulence de cellules VNC lorsqu'elles sont inoculées dans un hôte animal. Ces résultats ont entre autres été obtenus sur les pathogènes *Vibrio vulnificus* (Oliver et Bockian, 1995 ; Oliver *et al*., 1995), *Vibrio cholerae* O1 (Colwell *et al*., 1996) et *Salmonella typhimurium* (Caro, 1998). Cependant, ces études n'ont pas pris en compte la probabilité de présence de bactéries cultivables dans les inoculums et ne constituent donc pas des démonstrations définitives de l'aptitude à la reviviscence (Kell *et al*., 1998 ; Sylvester *et al*., 2001). Lors d'une étude où cette probabilité a été prise en compte, aucune reviviscence ou virulence n'a pu être attribuée à des cellules VNC de *Salmonella typhimurium* (Smith *et al*., 2002). Par contre, des expériences menées *in vitro* ont permis de mettre en évidence cette reviviscence de manière incontestable, en particulier sur *Vibrio cholerae* O1 et *Salmonella typhimurium* DT101 lors d'un choc thermique (Wai *et al*., 1996 ; Gupte *et al*., 2003), sur *Vibrio parahaemolyticus*, *Vibrio vulnificus* et *E. coli* O157 par ajout de catalase ou de pyruvate de sodium, qui sont des composés protégeant de l'effet du péroxyde d'hydrogène (Mizunoe *et al*., 1999 ; 2000 ; Bogosian *et al*., 2000). Enfin, l'effet d'un inducteur de croissance thermostable entérobactérien a pu être établi sur la reviviscence de *S. typhimurium* et *E. coli* enterohémorragique (Reissbrodt *et al*., 2002).

CONCLUSION

Pour certains auteurs, l'état viable mais non cultivable chez les bactéries dont les pathogènes normalement cultivables paraît être une réponse physiologique « programmée » de la cellule à certains stimuli. L'état VNC peut être considéré comme une forme de survie permettant à la cellule de mieux résister aux facteurs environnementaux stressants comme ceux des milieux marins-côtiers, dans l'attente d'apparition de conditions environnementales favorables, de « jours meilleurs » (Morita, 1993 ; Oliver, 1995 ; Kell *et al*., 1998). Ce phénomène de cellules bactériennes viables mais non cultivables nécessite d'entreprendre des études physiologiques et génétiques fondamentales pour mieux comprendre ce qui permet à une cellule cultivable de perdre ce pouvoir tout en restant viable (VNC), puis soit de réactiver son pouvoir de cultiver, soit de rester définitivement dans cet état et d'évoluer vers la mort. Aussi la présence de telles cellules pathogènes VNC dans les écosystèmes marins-côtiers devrait être prise en considération et conduire à des sujets de réflexion en santé publique (Barer *et al*., 1993).

Cependant, pour d'autres auteurs, l'état VNC ne serait qu'un « artefact » d'un état de dégradation cellulaire des bactéries soumises aux facteurs environnementaux stressants. Cette dégradation cellulaire ne peut conduire alors qu'à la lyse cellulaire et donc à la disparition de telles cellules (Desnues *et al.,* 2003 ; Cuny *et al*., 2005).

Les résultats contradictoires sur cette question de la virulence des cellules VNC de bactéries pathogènes sont sans doute à moduler en fonction des bactéries concernées. En effet, il se pourrait que des bactéries pathogènes allochtones aux systèmes marins comme *Salmonella* ne puissent se maintenir facilement dans ces milieux, et que leur devenir soit la dégradation cellulaire. Pour des bactéries autochtones comme les Vibrio, l'état VNC pourrait être une réponse adaptative à la période hivernale défavorable à sa multiplication.

Des études fondamentales sont donc à poursuivre pour mieux comprendre le phénomène d'apparition des bactéries viables mais non cultivables, de leur reviviscence éventuelle et, pour les pathogènes humains, du maintien de leur virulence.

Vibrio interactions with biotic and abiotic surfaces in seawater and links with pathogenicity

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ABSTRACT

Bacterial adhesion is a very important event in the colonization of animate and inanimate substrates and provides a link between the behavior of microorganisms in natural environments and their pathogenicity potential. In coastal and estuarine systems, marine snow, detritus, plankton and other substrates provide stable, localized microhabitats for colonizing bacteria. Adhering bacteria, in comparison to free cells, show differences in metabolic activity, morphology and size. A common feature among vibrios is the presence of multiple lifestyles: a planktonic, free swimming state and a sessile existence on a surface. These bacteria are spread in coastal aquatic environments throughout the world, being more common in warmer waters, adhering to different substrates, mainly chitin containing surfaces. Moreover, high concentrations of vibrios can be found inside filter feeding invertebrates that sieve suspended food particles from the aquatic environment. Multiple studies have been conducted to understand the mechanisms by which vibrios and, in particular, *Vibrio cholerae*, interact with the different hosts. In general, the adhesive process appears to be multifactorial, with contributions from a variety of different cell surfaces and secreted components. Some recent findings on both interactions with chitin containing surfaces and mussel hemolymph are briefly summarized.

INTRODUCTION

Survival in any particular ecosystem requires a microorganism to be equipped with a battery of adaptative response mechanisms to meet demands such as nutrient limitation, UV stress, temperature fluctuation, protozoan predation, viral infection and changes in salinity. Attachment to and colonization of biotic or abiotic surfaces is a common mechanism by which various microorganisms enhance their ability to survive in diverse environments (Fletcher, 1985).

Bacterial adhesion is a very important event in the colonization of animate and inanimate substrates, providing a link between the behavior of microorganisms in natural environments and their pathogenicity potential. In fact, it is well known that this phenomenon is a prerequisite for epithelial cell colonization by both indigenous and pathogenic bacteria. On the other hand, marine bacteria that fail to attach to surfaces present in the aquatic environment fail to flourish and are washed away. In coastal and estuarine systems, marine snow, detritus, plankton and other substrates provide stable, localized microhabitats for colonizing bacteria. Adhering bacteria, in comparison to free cells, show differences in metabolic activity, morphology and size (Roszak and Colwell, 1987a).

A common feature among vibrios is the presence of multiple lifestyles: a planktonic, free swimming state and a sessile existence on a surface (Pruzzo *et al*., 2005). These bacteria are spread in coastal aquatic environments throughout the world, being more common in warmer waters as shown by a large number of studies (Baffone *et al*., 2006; Huq *et al*., 1984). Multiple studies have been conducted to understand the mechanisms by which vibrios and in particular *V. cholerae* adhere to different surfaces, inside and outside the human host (Chiavelli *et al*., 2001; Reguera and Kolter, 2005; Watnick *et al*., 1999). In general, the adhesive process depends on contributions from a variety of different cell surfaces and secreted components.

VIBRIO CHOLERAE INTERACTIONS WITH CHITIN

Chitin is the most abundant polysaccharide in the aquatic ecosystem and the principal component of zooplankton exoskeleton. Chitin surfaces are colonized by autochthonous bacteria, mainly *Vibrio* spp. that, producing chitinase(s), are responsible for the degradation of chitin to soluble oligosaccharides. Without such bacterial activity that returns the insoluble polysaccharide to the ecosystem in biologically useful form, ocean waters would be depleted of carbon and nitrogen in a short time (Yu *et al*., 1987).

Different surface ligands such as TCP (toxin coregulated pilus), MSHA (mannose sensitive hemagglutinin), ChRP (chitin-regulated pilus), CBP (chitin binding proteins) and Gbp (N-acetyl glucosamnine-binding protein) seem to be involved in *V. cholerae* association with chitin containing surfaces (Chiavelli *et al*., 2001; Meibom *et al*., 2004; Reguera and Kolter, 2005; Tarsi and Pruzzo, 1999; Watnik *et al*., 1999) (Table 1). We have shown that bacterial membrane proteins, named "chitin binding proteins (cbps), play an important role in such interactions (Tarsi and Pruzzo, 1999). Up to now, cbps have been shown in at least three species: *V. harvey*, *V. alginolyticus* and *V. cholerae* (Montgomery and Kirchman, 1994; Carli *et al*., 1993; Tarsi and Pruzzo, 1999); in a forthcoming paper, a similar protein system is shown in *Vibrio nereis, Vibrio anguillarum, Vibrio splendidus, Vibrio metschnikovii, V. parahaemolyticus,* and *V. vulnificus*. Cbps may give vibrios an important advantage when competing with other marine microorganisms for this particular substrate, serving the function of fostering bacterial attachment.

Table 1. *V. cholerae* ligands involved in the interactions with different biotc and abiotic surfaces present in sea water.

V. cholerae ligand	Target surface				References
	M. galloprovincialis hemocytes	Cellulose	Chitin	Zooplankton	
TCP[a]	nt	-	+	-	Reguera and Kolter, 2005
MSHA[b]	+	+	+	+	Chiavelli *et al*., 2001; Pruzzo *et al*., 2005; Watnick *et al*., 1999
ChRP[c]	nt	nt	+	nt	Meibom *et al*., 2004
CBP[d]	-	nt	+	+	Kirn *et al*., 2005; Tarsi and Pruzzo, 1999
Gbp[e]	-	nt	+	+	

+ and -: presence and absence of the studied property.
nt: not tested.
[a]TCP: toxin coregulated pilus; [b]MSHA: mannose sensitive hemagglutinin; [c]ChRP: chitin-reglated pilus; [d]CBP: chitin binding proteins (outer membrane and secreted); [e]Gbp: GlcNAc-binding protein.

Several data have suggested that chitin made surfaces are ecosystems that may deter the onset of a nonculturable state or improve growth of these bacteria (Huq *et al*., 1984; Colwell, 1996). We have shown that during exposure in ASW for relatively long periods of time at 5°C and 18°C, *V. cholerae* O1 retains the ability to express cbp and to interact with chitin particles and copepods, though less efficiently than when cells are incubated at 35°C (Pruzzo *et al*., 2003).

Vibrio cholerae interactions with chitin are of great interest for human health for different reasons: (i) bacteria adhering to chitin-containing zooplankton organisms persist longer in the aquatic environments than free forms (Colwell, 1996;); (ii) *V. cholerae* biofilms that develop on single chitin containing plankton may rise to the level of an infectious dose (Colwell, 1996); (iii) zooplankton (mainly copepods) may represent an environmental reservoir of vibrios (Colwell, 1996); (iv) bacterial association with chitin increases the microbe's resistance to acids such as those secreted by the lining of the stomach, thus, if ingested by drinking contaminated water or improperly cooked shellfish, there is an increased possibility that the microbe will cause cholera (Nalin *et al*, 1979); (v) attachment to chitin induces the synthesis of the DNA-uptake apparatus, a phenomenon that contributes to bacterial genetic diversity, i.e. acquisition of virulence genes (Meibom *et al*., 2005).

Another important role in pathogenicity for bacterial capability to interact with chitin has been proposed more recently. In fact, recent studies by Zampini *et al*. (2005) later confirmed by Kirn *et al*. (2005), have supported the model of selection of binding proteins that mediate adherence to epithelial cells during *V. cholerae* colonization of chitinaceous surfaces. The fact that GlcNAc is a widespread compound that, besides being the constituent of chitin, is also present in complex carbohydrate structures of animal cell membranes, has suggested that *V. cholerae* may utilize the same ligand(s) to interact with GlcNAc-containing substrates present in the aquatic environment and in human intestine. To explore this possibility, Tn*pho*A mutants of *V. cholerae* O1 classical strain CD81 were investigated for their ability to attach to chitin-containing substrates (chitin particles and *Tigriopus fulvus* copepod exoskeleton) and to cultured intestinal epithelial cells. The results, confirming the complexity of *V. cholerae* adhesion process, showed that mutant lacking a CBP of approximately 53 kDa has a reduced capability to adhere to chitin particles, copepods and intestinal cells (Zampini *et al*., 2005); the same mutant was shown to be very poor at colonizing freshly isolated rabbit intestinal tract cells and to be less toxinogenic *in vitro* than the parent (Singh *et al.,* 1994). Studies performed by Kirn *et al*. (2005) have also shown that a *V. cholerae* O1 strain lacking a 52 kDa GbpA protein, localized both in the bacteria outer membrane and culture supernatant, has a tenfold reduced LD50 for mice than the wild-type strain. Moreover, passive immunization experiments conducted with antisera from rabbits immunized with a GbpA-His fusion protein provided a significant survival advantage to the mice.

Interestingly, all these studies strongly point to a common link between the persistence of some potentially pathogenic bacteria in the environment and infection of the human host, and suggest that virulence mechanisms of bacteria that have environmental reservoirs may represent a consequence of adaptive mechanisms to the environment (Reguera *et al*., 2005).

VIBRIO INTERACTIONS WITH BIVALVES

In sea water, vibrios, as well as other indigenous and non-indigenous bacteria, can be entrapped by filter feeding invertebrates that sieve suspended food particles from the aquatic environment. Filter feeding may result in concentration of potential pathogens that can either establish a commensal relationship with the host, without causing diseases, or proliferate and invade soft tissues, resulting in high mortality of bivalves. Moreover, accumulation of bacteria pathogenic to humans in the tissues of edible bivalves is of great concern to public health; in fact, consumption of raw or inadequately cooked bivalves has been implicated in numerous food poisoning outbreaks. Shellfish depuration in controlled waters is used extensively worldwide to decrease the number of unwanted microorganisms to acceptable levels for human consumption. Regarding this depuration procedure, bacteria behave differently; for instance, some *Vibrio* species have been reported to be resistant to the process and are able to persist and multiply within shellfish tissues (Murphree and Tamplin, 1992).

Persistence of bacteria in bivalves largely depends on their sensitivity to the hemolymph bactericidal activity. Bivalve hemolymph contains both the hemocytes that are responsible for cellular defense mechanisms (i.e. phagocytosis, production of reactive oxygen intermediates, and release of lysosomal enzymes) and humoral defense factors, such as opsonins and hydrolytic enzymes (Olafsen, 1995). Bacteria show different capacities to survive hemocyte phagocytosis as a consequence of the different ability to attract phagocytes, interact with opsonizing molecules, bind hemocytes, and survive intracellular killing (Canesi *et al*., 2005).

At present, data on the role of bacterial components in both favoring and inhibiting the above processes are scanty (Canesi *et al*., 2005; Harris-Young *et al*., 1995; Pruzzo *et al*., 2005). Recent data on surface components involved in interactions of *V. cholerae* El Tor with *M. galloprovincialis* hemocytes indicated that humoral factors might specifically opsonize MSHA-positive vibrios, *via* D-mannose-sensitive binding, enabling them to more efficiently adhere to hemocytes (Zampini *et al*., 2003); adherence is then followed by efficient internalization and killing. On the basis of previous data indicating that mussel hemolymph serum contains soluble factors that are involved in D-mannose-sensitive interactions between *E. coli* carrying type 1 fimbriae and hemocytes (Canesi *et al.,* 2005), it was hypothesized that broad-spectrum opsonizing molecules able to bind both MSHA and type 1 fimbriae may be present in hemolymph serum.

Studies on host-pathogen interactions in mammalian models of infection have widely demonstrated that a common strategy of different pathogenic bacteria to escape or overcome the defense response of the host is to undermine host cell functions through disregulation of its signalling pathways (reviewed by Rosemberger and Finlay, 2003). In particular, activation of MAPK (Mitogen Activated Protein Kinase) signaling is crucial for many responses to infection and represents a strategic target for infectious organisms. It was recently found that hemolymph bactericidal activity is less active toward wild-type *V. cholerae* in comparison to other bacteria (e.g. fimbriated *E. coli*) and that this phenomenon may be partly ascribed to the inability of the *Vibrio* strain to stimulate the stress-activated signaling pathways involved in activation of the bivalve host response (Canesi *et al*., 2005). Therefore, despite the presence of common mechanisms involved in surface interactions between hemolymph components and fimbriated *E. coli* and *V. cholerae* expressing MSHA, the *V. cholerae* strain apparently lacks cellular or secreted components responsible for triggering a signal transduction pathway crucial for full hemocyte activation. Alternatively, in analogy with the interactions between mammalian host cells and certain pathogens, specific *V. cholerae* products may lead to disregulation of the hemocyte host signal transduction pathways, by either interrupting kinase cascades upstream of MAPK activation or specifically activating dephosphorylation.

While investigating the interactions between vibrios and *M. galloprovincialis* hemolymph, the effect of hemolymph serum on *V. cholerae* adherence to cultured intestinal cells was also studied. It was found that *in vitro* persistence in bivalve hemolymph serum enhances attachment to human intestinal cells (unpublished results). These data suggest that bacterial colonization capability depends on both bacterial properties and environment-acquired factors and further confirm the link between the persistence in the environment and pathogenicity to humans.

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New biotechnology prospects under the Mediterranean Sea

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1. ABSTRACT

The discovery, in a deep-sea hypersaline anoxic basin (DHAB) of the Eastern Mediterranean, of few enzymes that do not fit into any previously defined category of protein superfamilies and exhibit unique structural features marks a significant step in chemical biotechnology, in particular regarding the synthesis of new chiral synthons, and opens new research in pharmaceutical and therapeutic properties.

2. INTRODUCTION

One of the most exciting current research endeavours is the exploration of biological and functional diversity, especially that existing in the extreme conditions that can occur at the limits of the biosphere (Hewitt, 2004; Dolan, 2005). There is a pervasive perception that undiscovered microbial diversity is enormous and is a treasure trove for new biotechnological applications (Whitman *et al*., 1998; Koonin *et al*., 2002). Defining the limits of metabolic diversity and microbial lifestyles on our planet will provide a tangible framework for the development of new applications for the future.

This will not only lead to discovery of unknown metabolic and physiological activities and molecular and cellular structures, and reveal the range of potential life forms on Earth, but also enable definition of the mechanistic basis of life under the most hostile conditions known for the biosphere.

Deep-sea hypersaline anoxic basins of the Eastern Mediterranean (DHABs) (Figure 1) represent unique, extreme, and largely unexplored habitats, over 3,500 m below sea level. They were created 5–6 million years ago by the dissolution of buried Messinian evaporitic deposits to form very stable brines entrapped in sea floor basins and sharply stratified from the overlying water column (Vengosh and Starinsky, 1993; Van der Wielen *et al*., 2005).

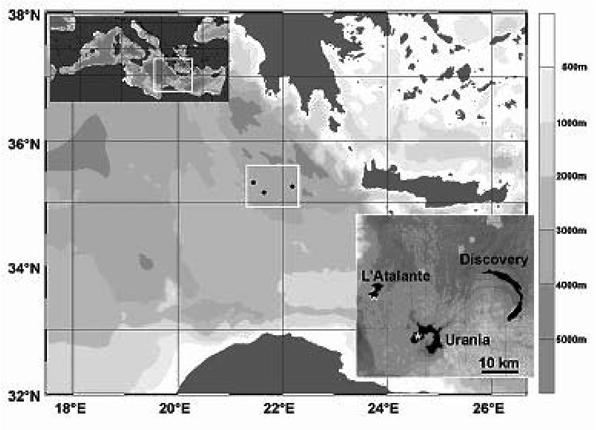

Fig. 1. Location of Three Deep Anoxic Hypersaline Basins in the Eastern Mediterranean Sea.

They are characterized by extremely high salinity and corresponding density, high hydrostatic pressure, absence of light, anoxia, and a sharp chemocline (Figure 2).

These physicochemical features have ensured that the DHABs have been physically isolated from other habitats on the planet for thousands (2,000–176,000) of years (Wallmann *et al*., 2002; Hübner *et al*., 2003), may have resulted in the selection of unusual organisms, and probably prevented their dispersal. They are expected therefore to yield novel microbial diversity and unknown cellular gene products with interesting properties.

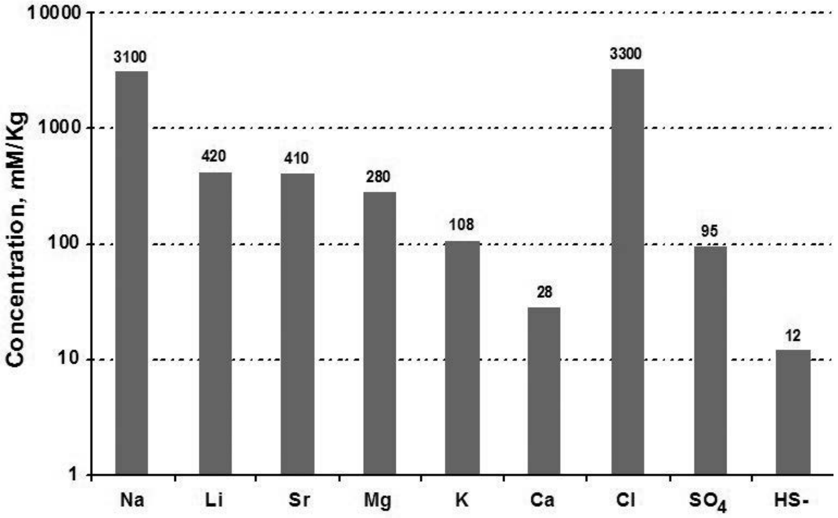

Fig. 2. Chemical composition of Urania basin brine. The concentration of major ions is given in mM/Kg (density of brine is 1.13).

The metagenome approach has opened access to a vast wealth of information in previously inaccessible organisms from unusual and extreme environments, in particular DHABs. In particular, we are interested in the identification and application of enzymes in biocatalysis which is especially important for processes to synthesize building blocks for pharmaceuticals and fine chemicals (Buchholz *et al*., 2005). Following this reasoning, we have recently posed the question: is new enzymatic diversity, exemplified by esterases, to be found in microbes present in DHABs, specifically in the brine:seawater interface of the Urania West Basin, one of five recently discovered DHABs in the Southeastern Mediterranean Sea (the others being the Bannock, L'Atalante, Discovery, and Tyro Basins)? (Ferrer *et al*., 2005).

We want to focus the attention on the fact that in the near future metagenomics may significantly contribute to the discovery of novel metabolic conversions and unravel the mechanisms of action of the proteins with yet unknown functions. This will provide a better understanding of the intriguing biology of *marine* microorganisms, especially those not amenable to the common culturing techniques, and how they can offer new advances/potential in public health.

3. RESULTS AND DISCUSSION

DHABs represent isolated, extreme environments that harbor thus far uncharacterized biodiversity. To explore the functional biodiversity of the seawater:brine interface of one such DHAB, the Urania West Basin was sampled during the BIODEEP-II cruise of the RV *Urania* in September 2001, and a subsample was immediately supplemented with sterile crude oil (Arabian Light, 0.5% v/v) to stimulate microbial growth and increase biomass. After incubation for 4 weeks at 15°C, total DNA was extracted from the culture, and a metagenome expression library in *Escherichia coli* was generated by using the bacteriophage lambda-based ZAP. At a first glance, the discovery of only five new esterases was achieved.

Our study of esterases mined from the Urania West Basin interface suggests that DHAB interfaces contain microbes and biomolecules displaying diverse functionalities that reflect life specifically adapted to the brine, to the water column and, most interestingly, to the interface. Two of the esterases showed relatedness to known esterases and exhibited no distinctive characteristics related to pertinent physico-chemical properties of DHABs. Thus, we assume that their genes originated from bacteria that inhabit normal *marine* habitats and that either were living on particulates that descended to the brine interface or were living on or close to the upper face of the chemocline. One enzyme also showed relatedness to known esterases but exhibited halotolerance, so may have originated from a brine-tolerant organism.

On the other hand, the last two enzymes were entirely new and exhibited unusual, habitat-specific characteristics, such as preference for or tolerance of high hydrostatic pressure and salinity, and, uniquely so far, exceptional stability and activity stimulation by polar solvents. We therefore suggest that they are DHAB interface-specific, and that these interfaces are the specific habitats of the microbes that produce them.

We further discovered that one out of five enzymes (O.16) exhibits rather surprising properties: First, it efficiently resolves solketal acetate (Figure 3) -a chiral building block considered to be an important intermediate for therapeutic drug development, e.g. for AIDS- for which no sufficiently selective enzyme has been identified. Therefore, our results in DHAB enzymes suggests that *marine* metagenomics will increasingly contribute to medical advances, delivering new immunomodulators, antibiotics and therapeutic agents to prevent and cure diseases and will provide new complements with specific human condition/health defensive functions.

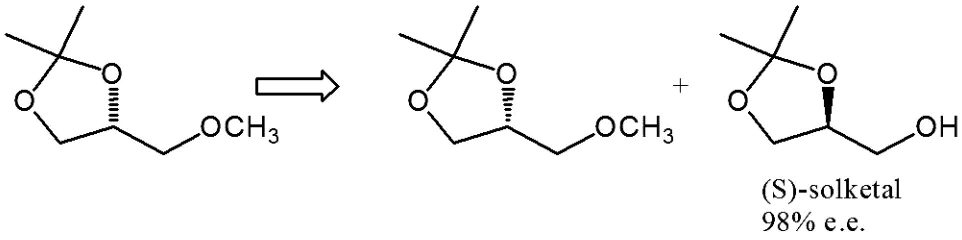

Fig. 3. DHAB enzymes are good candidates for drug production. Kinetic resolution of racemic solketal acetate with O.16 esterase proceeds with the highest enantioselectivity reported so far in the literature.

A much more striking feature relates to its unique sequence and higher level of structural and functional complexity (Arpigny and Jaeger, 1999): this is the first enzyme of this class to contain *three* differentiated catalytic sites (Figure 4). These features have evolved to assure catalytically powerful functionalities over the wide range of physico-chemical conditions prevailing in the *marine* water column, the steep gradients of physico-chemical conditions at seawater:brine interfaces of DHABs, and the highly stressful extreme conditions of DHAB brines. We also suggest that this structure represents a specific adaptive substrate-scavenging-strategy to allow microbe survival in the substrate-poor *marine* environments like deep sea Mediterranean anoxic hyper saline lakes.

As far as we know, esterases mined from the metagenome library of the Mediterranean Urania Basin hypersaline brine interface have unusual structures and superior catalytic properties to other known esterases, and they therefore may be candidates for the synthesis of optically pure compounds and pharmaceutical intermediates.

In order to gain insight into the microbial functions that are relevant to DHABs, it is necessary to identify DHAL-associated genes/enzymes, and their functions, in order to be able to formulate testable hypotheses on how such activities/functions may affect the existence of potential unique life forms that may provide the development of novel diagnostic tools and/or products that could direct preventive or therapeutic approaches to enhance health and chronic inflammatory diseases.

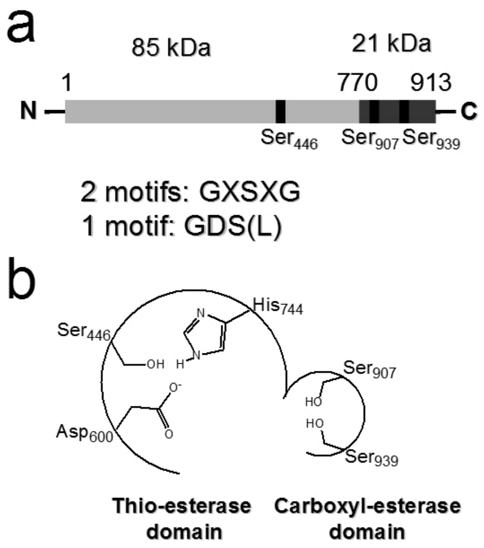

Fig. 4. Structural features: O.16 contains 3 catalytic serines mediating different activities in two domains: (a) schematic representation of the two domains (in clear grey, thio-esterase domain; in dark grey, carboxylesterase domain) (a) and location of catalytic serines (b).

Infections is mainly a problem of morbidity and economic costs (considerable medical care and lost work productivity), with poorer, non-industrialized nations being the most affected. The understanding of microbial communities in *marine* products will have an important impact for the public health system, since many infections and inflammatory diseases require the development of novel therapies. The discovery of novel *marine* bioactive metabolites, proteins and/or enzymes may resolve the large economic and health burden in both industrialized and developing countries. Our goal, in the future is not to gain a comprehensive overview of the meta-genomics of DHAB but rather to conduct a pilot study, in anticipation of a more comprehensive analysis, by characterizing proteins/genes with novel public health functions.

4. SIGNIFICANCE

We have shown that the activity-based mining of "metagenome" libraries of microbial genetic resources from *marine* ecosystems resulted in the discovery of novel enzymes. Esterases mined from the metagenome library of the Mediterranean Urania Basin hypersaline brine interface have unusual structural signatures incorporating three catalytic active centers mediating distinct hydrolytic activities, adaptative tertiary-quaternary structures, and superior catalytic properties, and are therefore new candidates for the synthesis of optically pure compounds and pharmaceutical intermediates. If, as we suggest here, these esterases serve as proxies of other enzymes and metabolic activities of the microbes of the DHABs, then a significant amount of new diversity is waiting to be discovered in such brines and their interfaces.

5. FUTURE PROSPECT OF MARINE SCIENCE AND PUBLIC HEALTH NETWORK

It is estimated that *marine* ecosystems, including the subsurface, harbour $\sim 3.67\times 10^{30}$ microorganisms that represent an extraordinary and dynamic gene pool of biodiversity (Li and Qin, 2005). The extreme variety in *marine* environments of pressure, salinity, temperature and nutrients enable *marine* microorganisms to develop unique biochemical and physiological competence for survival. At that point, one area of particular interest in the 21st century is the development of new types of antibiotics to cure rapidly appearing multidrug-resistant pathogens and newly emerged infectious agents for which the current arsenal of drugs is quickly and overwhelmingly outgunned. *Marine* chemo-diversity is one of the targets for searching for natural drug products, and enormous research resources are being dedicated to this field to fulfil the ever-growing urgency and demand for new antibiotics and more effective medicines for deadly diseases, such as cancer, and cardio-vascular diseases. One might wonder why the number of commercially-available drugs of *marine* origin falls far behind those derived from culturable soil organisms, which provide 60% of the antibiotics in the pharmaceutical industry. There are at least three obstacles for reaping the fruit of *marine* chemodiversity in comparison to the chemical richness of culturable soil microbes. First, most *marine* microorganisms that produce bioactive compounds cannot be cultivated using traditionally employed cultural conditions despite recent progress in cultivation technology; for the few that can be cultured, the culturing methodology is time-consuming. Second, the bioactive compounds of *marine* origin are usually present in trace amounts in *marine* ecosystems, which make it difficult to obtain the quantities required to facilitate even the minimum requirements for preclinical and clinical studies. Third, *marine* chemicals often have unique structural characteristics for which there is, in many cases, a lack of efficient and economically viable ways to chemically synthesize these compounds or ways to produce the chemicals in the large quantities required for drug evaluation and downstream clinical trials.

As an alternative, we believe that functional metagenomics of *marine* ecosystems could provide informative answers and solutions to overcome the cultural barriers, as has been the case in the successful investigation of diverse human-health-active metabolites produced by microbes in terrestrial ecosystems. We believe so because it is generally accepted that *marine* microbial communities account for more than 80% of life on earth and have an indispensable role in primary energy and carbon recycling; moreover, our recent investigations in novel enzymes from deep-sea hypersaline anoxic basins of the Eastern Mediterranean (DHABs) suggest that a significant amount of new diversity is waiting to be discovered in such brines and their interfaces. The genomic libraries generated using metagenomic approaches would provide important resources for our own health.

Acknowledgements

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Arsenic: is it worth monitoring in the Mediterranean Sea?

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Arsenic (As) is a widely distributed element in the marine environment. Its sources are both natural (e.g. geothermal activity) and anthropogenic (e.g. industrial processing of phosphate rocks, bauxite and non-ferrous metals such as Cu, Zn, Pb, Au, Co, and fossil-fuel burning). Arsenic is also present in a wide variety of compounds used in pesticides, fertilisers, food additives, paints, glass, alloys, medicines, electronic components and wood preservatives.

Annual world production of arsenic reaches about 30,000 tons. Most of which is eventually released into the marine environment through runoff from land and river basins, as well as via air deposition (Phillips, 1990; Neff, 1997). Atmospheric inputs apparently contribute little to the global arsenic budget of the ocean. Major sources of arsenic in surface waters of the ocean are riverine inputs and upwelling of deep ocean water enriched in arsenic. Hence, the contribution of human activities to the arsenic budget may be important in estuaries and coastal waters.

The general concern regarding arsenic in the environment is mainly due to the widely-held view that it is highly toxic. Indeed, arsenic has historically been considered by the public as the archetype poison and is represented as such in many novels and plays.

During the last decades, a substantial body of evidence has shown that arsenic may cause severe poisoning (arsenicosis) leading to cardiac and neurological disturbances, skin, liver and lung cancers, and eventually death. Exposure of large human population to drinking water containing excessive concentrations of arsenic does occur in many countries of the world (e.g., Smedley and Kinniburgh, 2002; Visoottiviseth *et al*., 2002; Farias *et al*., 2003). This issue is of particular concern in poor regions of the world with limited access to drinking water such as Bangladesh or West Bengal where 40 millions people are drinking arsenic-contaminated water. In Southern Thailand, it has been shown that waste piles from tin-mining contain very high concentrations of arsenic as arsenopyrite. Leaching from these piles is contaminating local soil and groundwater serving as a water source for the local population, exposing in only the Ron Phibun District more than 30,000 people to chronic arsenic poisoning, with more than 1,000 cases of severe arsenicosis observed in 1996 (Visoottiviseth *et al*., 2002).

From the literature related to mammals, it is now well known that arsenic can compete with inorganic phosphate during the phosphorylation of adenosine diphosphate (ADP) to adenosine tri-phosphate (ATP), leading to uncoupling of oxidative phosphorylation, mitochondrial impairment and inhibition of energy metabolism. More recently embryotoxicity and genotoxicity have also been reported for arsenic-containing compounds, with effects involving e.g. oxidative DNA damage, DNA protein crosslinks and inhibition of DNA-repair enzymes (see e.g., Fattorini and Regoli, 2004).

Although toxicity mechanisms of arsenic in marine organisms are much less documented than in terrestrial mammals, the bioaccumulation potential and the sensitivity of these organisms towards arsenic are well known.

Chemical speciation of arsenic in aquatic environments is the most important factor determining the accumulation of the element by biota and its toxicity. Indeed, arsenic toxicity is strictly related to its chemical form. Inorganic arsenite, As(III), and arsenate, As(V), are the most toxic species; methylated compounds such as methylarsonate, dimethylarsinate, trimethylarsine oxide and tetramethylarsonium are considered as moderately toxic, whereas complex organic compounds including arsenobetaine, arsenocholine and arsenosugars are considered non-toxic to living organisms (Figure 1) (e.g., Ellwood and Maher, 2002; 2003).

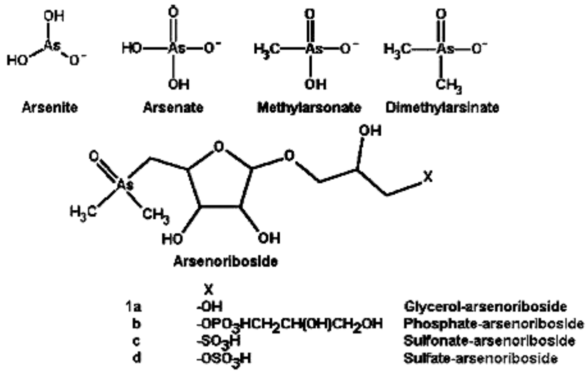

Fig. 1. Structure of some arsenic compounds found in the environment (from Tukai *et al.,* 2002).

In the marine environment, inorganic forms of arsenic predominate in seawater and sediments, whilst it is generally reported that marine organisms bioaccumulate the element mainly as organic, non-toxic compounds. Several authors have suggested that these organic forms would be the final products of detoxification processes of bioaccumulated arsenic (e.g., Phillips, 1990; Francesconi *et al*., 1998). However, important variation in concentrations and distribution of chemical forms of arsenic can be observed in marine organisms, often reflecting their trophic position or their ability to biotransform arsenic (Wrench *et al*., 1979; Fattorini *et al*., 2006).

Quantitatively, the most important routes of exposure of humans to arsenic are represented by inhalation of industrial dusts (particularly those from nonferrous metal smelters and coal-fired and geothermal plants) and consumption of drinking water and food, particularly seafood which is well documented to dominate total arsenic intake by humans (e.g., Phillips, 1990; Neff, 1997). The maximum acceptable human intake of arsenic in food has been set by the World Health Organisation as 2 $\mu g\ kg^{-1}$ body weight d^{-1} (equivalent to 160 $\mu g\ d^{-1}$ in a 80-kg person) (WHO, 1984). Nevertheless, since arsenic in seafood is present mainly as organic forms which are relatively non-toxic compared to inorganic arsenic and easily eliminated (short residence time), the risk associated with seafood consumption is generally considered as relatively low.

However, contrasting with the general trend of arsenic occurring in marine organisms mainly as organic arsenic, there is now a body of evidence showing that different organisms are able to accumulate arsenic up to unusual, very high levels and/or as the more toxic forms (e.g., Fattorini *et al*., 2005). This is for example the case for several polychaetes, such as the cirratulid *Tharyx marioni* which displays extremely high body concentrations of total arsenic (2,000 $\mu g\ g^{-1}$ dry wt; Gibbs *et al*., 1983) or *Arenicola marina* which accumulates arsenic predominantly (80%) as arsenate (Geiszinger *et al*., 2002). Elevated levels of arsenic (> 1,000 $\mu g\ g^{-1}$ dry wt), mostly present as dimethyl-arsinate, have been detected in the branchial crown of the Mediterranean fan worm *Sabella spallanzanii*, suggesting an antipredatory role for this relatively toxic arsenic compound (Fattorini and Regoli, 2004). Recent experiments also revealed the capability of this polychaete to produce this molecule through both methylation and de-methylation reactions (Notti *et al*., in press).

Similar observations have been reported in macroalgae. Some taxa such as the *Sargassum* sp. are known to contain high percentages (> 75%) of inorganic arsenic (e.g., Phillips, 1990).

More recently, very high arsenic concentrations (500 µg g^{-1}dry wt) were monitored in muscles of edible fish from the Bay of Cienfuegos, Cuba (Fattorini *et al*., 2004). These levels were recorded a few weeks after this semi-enclosed bay experienced an episode of acute arsenic contamination in December 2001 due to the accidental release of 3.7 tons of arsenic (as arsenate oxides) from a local nitrogen fertilizer factory situated near Cienfuegos City (150,000 inhabitants) (Figure 2). Since marine organisms are an important component of the diet of the population of Cienfuegos Bay (up to 116 kg fish yr^{-1} $person^{-1}$), local fisheries were rapidly prohibited by the governmental authorities and restored only when acceptable arsenic levels were again reported for seafood and fish. Although high arsenic concentrations could have been expected in this particular situation, the most noteworthy observation concerned the arsenic speciation found in certain edible fish studied (*viz*. *Caranx latus*, *Lutjanus synagris*). In fact, the proportion of inorganic arsenic in the fish reached 98% of the total arsenic load (Fattorini *et al*., 2004) which would result locally in a daily consumption of inorganic arsenic > 400 µg d^{-1} $adult^{-1}$, *viz*. more than twice the maximum permissible daily intake recommended by the World Health Organisation.

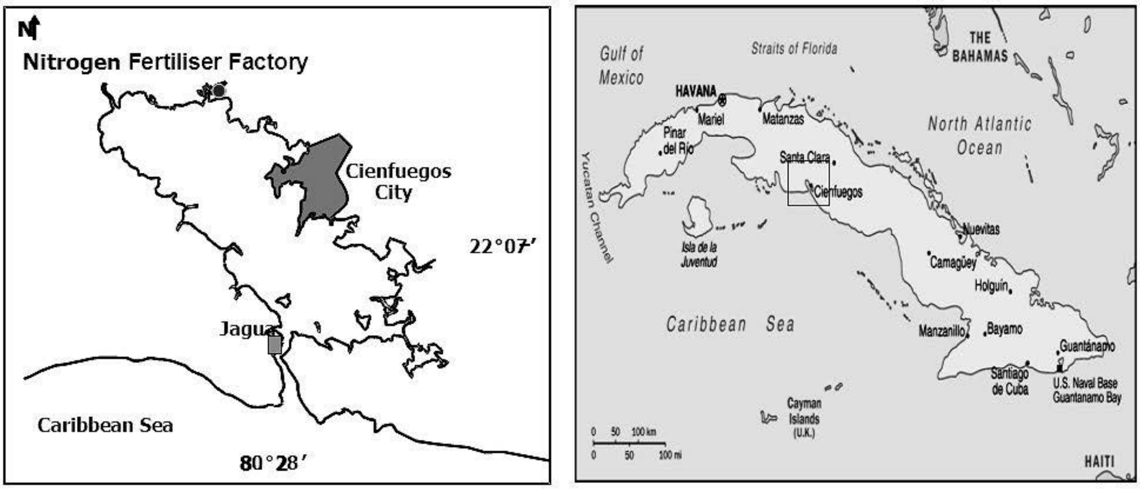

Fig. 2. Cienfuegos Bay, Cuba and location of the nitrogen factory responsible for the As spill in December 2001.

Environmental conditions and/or anthropogenic contamination are known to interfere with bioavailability of arsenic, and potentially influence the relative amounts of toxic forms in tissues of exposed organisms (e.g., Fattorini *et al*., 2004). For instance it is well known that an increase in N:P ratio in seawater greatly enhances the bioavailability of arsenic to phytoplankton (see e.g., Phillips, 1990). This in turn may lead to enhanced trophic transfer of arsenic to filter-feeders and their predators, and possibly to humans consuming the latter. For example, populations of the edible clam *Gafrarium tumidum* inhabiting non-contaminated areas of the New Caledonia lagoon are typically characterized by very high concentrations of total arsenic, up to 450 µg g^{-1} dry wt, (Hédouin and Warnau, unpublished). These levels were by far higher than those recorded in individuals from moderate to highly contaminated zones and are attributed to the influence of nitrogen inputs in coastal seawater due to terrestrial leaching of fertilisers used for local agriculture.

Overall, due to the complex biogeochemistry of arsenic and the related implications for its bioavailability and toxicity to seafood, fish and their human consumers, it is important to monitor levels of arsenic in the coastal marine environment. It is particularly important to discriminate between toxic and non-toxic chemical species of this element.

The different factors that can affect contamination levels and speciation of arsenic (natural and anthropogenic sources, water oligotrophy) and its toxicity discussed above are all occurring within the Mediterranean basin (e.g., non-ferrous mining industries widely distributed along the Mediterranean coastline, bauxite processing in South of France, geothermal activity in the Bay of Naples and in Sicily, phosphate rock processing along north African coasts, cyclic eutrophication of coastal waters due to summer tourism, etc.). Therefore, as shown in the examples above, these factors (and their interactions) could result in environmental levels and/or speciation of arsenic which differ locally from the typically harmless, expected ones. At present, these possibilities can not be disregarded since field data for arsenic in the Mediterranean Sea are missing for numerous areas, especially those along the southern and eastern coasts.

Although the Mediterranean coastline is composed of contrasting regions with important cultural, political and regulatory differences, it is nevertheless characterized by a relative geographical homogeneity regarding the use of the coastal zone. Indeed, the tourism-related economy, welfare and fisheries of human populations living around the Mediterranean rely substantially on the quality of Mediterranean waters. Hence, it is of prime concern to survey and ensure the quality of the Mediterranean coastal waters, and monitoring the levels of key contaminants including arsenic in seafood is therefore needed as well as requested by the regulatory bodies.

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CIESM Mediterranean Mussel Watch Program Phase II: towards an increased awareness of marine environment and seafood quality

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The oceans are a prime source of food for mankind. Contamination of seafood by radionuclides and toxic contaminants represents a serious threat to human health and a major economic concern.

In order to protect the health of seafood consumers, many countries have set guidelines for the maximum permissible levels of toxic pollutants in seafood (e.g., European Communities, 2001). Although such guidelines are essential for public health protection, other problems arise when guidelines are not met, which may lead to dramatic economic impacts on people and companies making their living out of sea resources. It is therefore of prime importance to monitor the levels of key contaminants in the coastal zone, where most fishery and farming activities are carried out. This is especially true for bivalve molluscs, such as mussels, oysters and scallops. While fishing and farming of these organisms represents a multi-billion Euro worldwide industry (Ifremer, 2002), these organisms may easily become unsuitable for human consumption due to their well known ability to accumulate very large amounts of pollutants even in scarcely contaminated environments. Measuring and monitoring pollutant levels in organisms, i.e. *bio-monitoring* these levels, represents the only method that takes into account and integrates all those changes in the water quality which may influence the accumulation of pollutants in seafood and, ultimately, their possible transfer to mankind via seafood consumption.

Monitoring programs were historically conceived to control the quality of the marine environment without special emphasis on how contamination of the media could potentially impact the health of edible organisms and mankind. However, during the past few decades there has been increasing evidence of the existence of a deep, mutual relationship between the health of the environment and that of mankind. Strict environmental monitoring activities have thus adopted an ecosystem-based approach, and "Mussel Watch" programs have been developed following this innovative concept.

The first Mussel Watch program was implemented in the USA during the mid 1960s by the US-EPA (Goldberg, 1975) in order to monitor levels of heavy metals and organochlorines, and more recently those of several biochemical parameters in marine coastal waters. Nowadays, the US Mussel Watch is coordinated by NOAA and has been extended to Latin America in the early '90 (Tripp *et al*., 1992). The bivalve species used to biomonitor these regions are the blue mussel

Mytilus edulis and the Californian mussel *M. californianus* along the North Atlantic and the Pacific coasts, and the American oyster *Crassostrea virginica* in the Gulf of Mexico and Southern Atlantic (Cantillo, 1991).

Several European countries have implemented similar national Mussel Watch programs (e.g. France, Italy, Spain). For instance, in France IFREMER developed the "Réseau National d'Observation (RNO)" in 1974 to assess the levels of metals, organochlorines, and PAH along the French coastlines. Biota (the mussels *M. edulis* and *M. galloprovincialis* and the oyster *C. gigas*) are collected twice a year in about one hundred sampling sites (passive biomonitoring) (RNO, 2005). In a second phase, the "Réseau Intégrateur Biologique (RINBIO)" has been implemented in 1996 to monitor the concentrations of chemicals and radiochemicals in organisms caged for several weeks prior to collection (active biomonitoring) (Andral *et al.*, 2004).

Despite the presence of several ongoing national programs, no large-scale Mussel Watch network was coordinated at the Mediterranean level until 2002, when CIESM developed and implemented a regional "Mediterranean Mussel Watch (MMW)", using the mussel *M. galloprovincialis* as a bioindicator species (CIESM, 2002a).

The CIESM MMW program was originally conceived with two main objectives: a) to develop an international trans-Mediterranean infrastructure of cooperating scientists and laboratories aiming to monitor levels of selected contaminants in coastal waters; b) to document reliable baseline levels of radionuclides in the Mediterranean and Black Sea coastal waters. Some 20 marine environmental laboratories from 15 different Mediterranean countries are now involved in this international, dynamic network, the first in the region specifically designed to monitor radionuclides before its extension to other selected pollutants. A major step was achieved by the end of 2004, when a specific reference material (AIEA-437) was used to carry out a large inter-comparison exercise across the entire Network. The production and testing of this external standard was financially supported and coordinated by IAEA-MEL and IRSN. Currently, samples from about 50 sites have been analyzed. On the basis of these results, the MMW network has been able to produce the first distribution map of Cesium-137 at the regional level.

After the successful outcome of the Phase I of the MMW program, it is now time to undertake Phase II. This second stage foresees the extension of the survey and monitoring activities to additional radionuclides and selected trace- and emerging-contaminants. In addition, the network will be extended to five additional Mediterranean countries in order to guarantee the monitoring coverage of the entire basin (Figure 1).

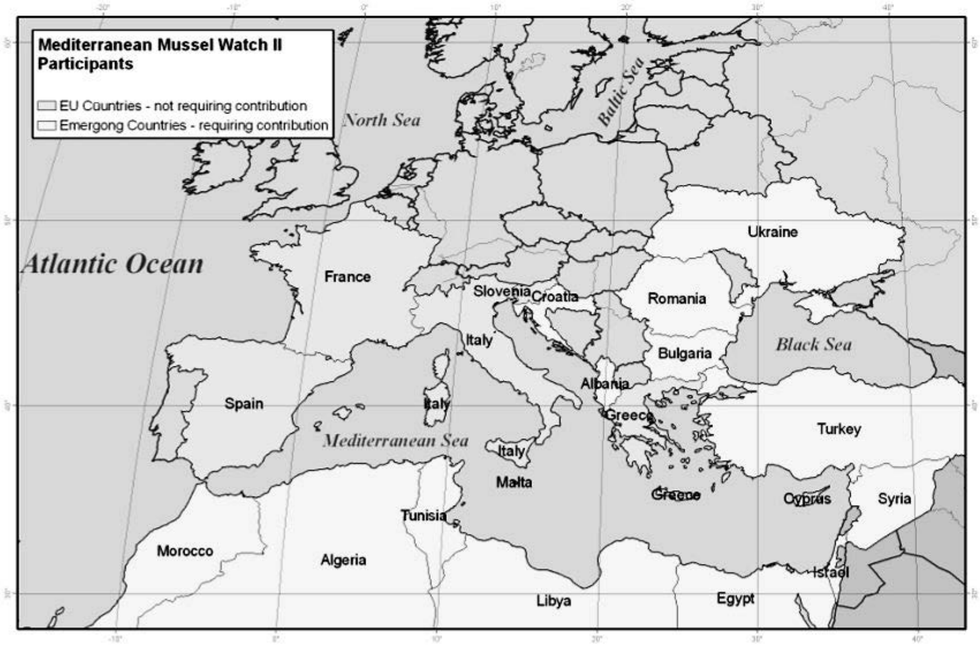

Fig.1. Countries participating to the Mediterranean Mussel Watch II project.

Trace contaminants such as arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), zinc (Zn), silver (Ag) and mercury (Hg) are naturally occurring elements ubiquitously found in the marine environment; however, human activities, such as industry, agriculture and mining, can dramatically increase their levels in all the abiotic and biotic compartments of the marine environment. Polonium-210 (Po-210) is a radioactive element that occurs naturally in very low concentrations in the marine environment, but due to its use in static eliminators and its occurrence as by-product of phosphate fertilizers and phosphoric acid industries, Po-210 represents a potential health hazard in many marine coastal areas. In recent years, the presence in the marine environment of many chemical and microbial constituents that were not historically considered as contaminants has been increasingly reported (e.g. new generation pesticides, pharmaceutically active compounds, antibiotic, emerging pathogens). These *emerging contaminants* cause known or suspected adverse ecological and(or) human health effects (Alvarez-Cohen and Sedlak, 2003).

Therefore, the main goal of the Mediterranean Mussel Watch II (MMWII) is to exploit its well established scientific international network to assess the extent and severity of contamination of the coastal waters of the Mediterranean Basin with respect to the above mentioned chemicals, with special emphasis on emerging contaminants. Indeed, after its international workshop convened in 2004 to summarize the current state of the knowledge on the problems arising from new kinds of contaminants (CIESM, 2004), CIESM is now ready to pioneer this novel research activities along the Mediterranean coastal areas.

Measurements of chemicals in the marine environment are often subject to large errors both in terms of precision and accuracy. Therefore, one fundamental effort will be devoted to enable a constructive use of the data collected in the present program by reducing these errors to such a degree that the data from the different Institutions involved a) be comparable among each others and b) realistically reflect the status/changes of Mediterranean coastal waters. For this purpose, the MMWII will be subject to a strict Analytical Quality Control Service (AQCS), which will include: (1) an inter-comparison exercise involving all the laboratories of the Network; (2) the routine analysis of selected reference materials (NIST-SRM 2976 for the mussel and IAEA-433 for the sediment) by each participant laboratory.

Within the framework of the program, an active collaboration (expertise and/or funding) with IAEA-MEL, WHO (Geneva) and MED-POL will be developed.

Results obtained from Phase Two will reliably map the current distribution of Polonium-210 and that of the selected trace- and emerging-contaminants in wild and transplanted mussels across the entire Mediterranean Basin. The scientific outcomes of the project will be published in international scientific journals, and be made accessible to broader audience as well. All data produced by the participants will be synthesized in a CIESM database, where they will be fully available on-line to all concerned parties. This unique dataset will provide critical information on the particular issue of seafood safety for human health, fishing and aquaculture.

Latest prospects in coastal pollution monitoring via remote sensing of ocean colour

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ABSTRACT

Remote detection enables the acquisition of large volumes of data in particular in areas which are not accessible. This paper describes shortly the main use of remote sensing for oceanography and marine applications. Then it presents a study related to lagoon pollution based on the Medium Resolution Imaging Spectrometer (MeRIS) which was launched in March 2002 and has been providing images since June 2002. Before its launch, we had implemented a method to improve its resolution by merging its images with Landsat Enhanced Thematic Mapper images in order to preserve the best characteristics of the two images (spatial, spectral, temporal). We now present the results of this method for real MeRIS images (level 1b and 2) in a coastal area. The robustness of the method is studied as well as the influence of the delay between the acquisition of the two images.

1. INTRODUCTION

Remote detection enables the acquisition of large volumes of data on the world's oceans and coastal regions, allowing scientists to observe events in remote and often inaccessible regions. These data can then be used for a variety of applications, such as mapping ocean dynamics and monitoring coastal change. These two functions-ocean modeling and coastal monitoring are either deployed directly or integrated in order to forecast changes in the phenomena detected and to optimize action plans. Such phenomena include oil slick drift, assessing changes in coastal pollution and the proliferation of algae.

One particular aspect of ocean and coastal phenomena modeling is the monitoring of lagoon pollution episodes. Alcatel Alenia Space, IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer, IRD (Institut de Recherche pour le Développement), the University of Bourgogne and Ecole des Mines have teamed up to demonstrate the potential capabilities of satellite imaging applications in this area. This type of pollution has a direct impact on shellfish farming and thus on human health. Modeling phenomena over long periods, forecasting pollution episodes, rapidly detecting known pollution and tracking its change are priority objectives for the agencies responsible for managing coastal zones. We worked closely with these satellite data users to conduct the study presented in this document.

Ocean forecasting

Oceanography uses data collected via remote detection to determine the direction and strength of currents, the amplitude and direction of surface winds and to measure sea surface temperature. Operational monitoring and forecasting, which is the focus of the integrated European marine environment and security program, MERSEA (coordinated by IFREMER), leverages the significant advances made in satellite altimetry, notably the TOPEX/POSEIDON and Jason altimeter radars which measure wave height and enable high-precision observation from space of changes in sea levels. These data are integrated with *in situ* surface and depth measurements into models that constitute regional and global "snapshots" of ocean change. Operational oceanography, which provides data on all aspects of the Earth's oceans, also uses other types of space data: scatterometers, which measure wind speed and direction, synthetic aperture radars, which measure changes in sea surface roughness due to winds and currents, as well as active radiometers and microwave sensors, which provide ocean surface temperature data.

Fig. 1. Jason in Alcatel Alenia Space clean rooms.

Ocean and coastal surveillance

Remote detection applications for ocean and coastal surveillance activities include: pollution detection (oil slicks, watershed discharge, water quality monitoring—with regard to farming, bathing, protected zones, etc.), assessment of phytoplankton concentration and algae proliferation, inventory and monitoring of aquaculture, evaluation of resources, definition of fishing quotas and monitoring of fishing practices, maritime traffic tracking (density, protected zones, etc.), coastal cartography (changes in coast lines**,** beach dynamics), protection of coastal regions, and the impact of anthropogenic activities.

Spatial optics and radar are the major components in ocean and coastal surveillance. Very high-resolution (superspectral and hyperspectral) data is required to measure the color of oceans. Furthermore, extensive spatial coverage provides a synoptic view of the distribution and spatial variability of chlorophyll (phytoplankton), water temperature, and concentration of suspended matter. Hyperspectral data provided by such instruments as MeRIS or MODIS (gathered in several narrow and near-infrared bands of the visible spectrum) allow enhanced precision in the classification of objects under study. For example, hyperspectral data are used today to obtain the cartography of regional time and spatial models of ocean color. The color of the ocean is determined by water quality, with chlorophyll one of the main indicators. Monthly and seasonal images are required for comprehensive modeling.

Hyperspectral data also provides information on water turbidity, bottom bathymetry and cartography in coastal zones.

Radar data provide additional information during nighttime observation or cloudy weather when optical data is not available. Radar data are also used in a wide range of applications, especially for detecting marine pollution. Oil slicks, for example, inhibit ocean waves, creating a surface which is smoother than the surrounding water. A weaker signal is returned to the imaging radar, producing a black line on the image. The direction of the slick and its predicted drift are determined using the operational oceanographic data described above, along with the meteorological data integrated into drift models in order to optimize rescue and clean-up operations. In addition, high-resolution radar imaging instruments enable the detection of polluting ships (identified as a lighter spot on the image).

Images captured by satellite sensors harbor huge potential for a rich variety of applications. Among them, pollution monitoring is of particular interest. Users of satellite images already benefit from a broad array of regular and long-term services. From a global perspective, the combination of satellite data and information gathered by airborne or ship-borne *in situ* sensors and integrated into models today makes it possible to provide a pertinent response to ocean management.

At the same time, further progress can be achieved. The burgeoning array of technical advances in spatial sensors, coupled with their improved revisit rate, should enable the development of additional applications to provide even more sophisticated services.

2. OCEAN COLOUR MONITORING

OCEAN colour monitoring is usually based on optical remote sensing with spatial resolutions around 1 km. This spatial scale is available with such sensors as NOAA’s Avanced Very High Resolution Radiometer, the Polarization and Directionality of the Earth’Reflectances Instrument, or Sea-viewing Wide Field-of-view Sensor, which cover one to several thousand kilometers in a single swath and provide very short revisit periods. The Medium Resolution Imaging Spectrometer (MeRIS) sensor, launched on board ENVISAT in 2002, was designed for sea colour observation, with a 300-meter spatial resolution, 15 programmable spectral bands and a three day revisit period. Three hundred meter is a high resolution for an oceanographic sensors but it is still too rough for coastal water monitoring, where physical and biological phenomena require better spatial resolution (Minghelli, 1999). On the opposite, multispectral Landsat Enhanced Thematic Mapper (ETM) images offer a suitable spatial resolution, but have only four spectral bands in the visible and near infrared spectrum, allowing poor spectral characterization. A few years ago, in order to combine the spectral resolution of MeRIS and the spatial resolution of Landsat ETM, we implemented a merging method proposed by Zhukov *et al*. (1999). Before the launch of ENVISAT, we applied this method to simulated MeRIS images (Minghelli-Roman *et al*., 2001).

This method is now applied to real MeRIS images. Two product levels are considered. Level 1b contains radiance measurements at the top of the atmosphere for the calibrated and geocoded fifteen (15) MeRIS bands. Level 2 contains normalized surface reflectance and several geophysical and biophysical parameters such as algal pigment index, suspended sediment, Rayleigh-corrected Vegetation Indices, aerosol type, cloud albedo. The method was tested for radiance (level 1b) and reflectance (level 2) over a coastal area of approximately 30x30 km^2 located around the Thau lagoon (southern France).

The main steps of the method are briefly recalled and the results are presented for levels 1b and 2. A validation method is proposed based on a statistical quality criterion, namely, the ERGAS parameter. In a previous paper we had suggested that the delay between the two images could have an impact on the fusion relevance in case of landscape evolution. This constraint is analysed as well. Eventually, the potential and limitations of this resolution improvement approach are discussed.

3. MeRIS – ETM MERGING METHOD

ETM images have high spatial resolution and low spectral resolution compared to MeRIS images. A MeRIS-ETM merging method was presented in detail by Minghelli-Roman *et al.* (2001). First, the MeRIS image is geometrically co-registered with the ETM image. Then, a multispectral classification is applied to the ETM image in order to divide its pixels into N_c classes. The only request is that all pixels must belong to a class (i.e. no pixel remains unclassified).

Each MeRIS pixel covers 100 pixels of the ETM classification. The proportion of each class is computed within each MeRIS pixel. For each class, a mean spectral profile is obtained by solving an algebraic system. The last step consists of substituting each classified pixel with its corresponding spectral profile. The output image is a 30 m spatial resolution (ETM spatial resolution) and 15 spectral bands (MeRIS spectral resolution).

For MeRIS level 2 images, we noticed that "black pixels" were located on land-water borders. Since atmospheric corrections use different methods on land and water, these mixed pixels are not corrected and their MeRIS level 2 reflectance is set to zero for all spectral bands. These pixels are used neither to solve the algebraic system nor to determine the spectral profile of the class. In order not to leave black pixels in the resulting image, we replace the classified ETM pixels by the spectral profile obtained for the pixels belonging to the same class.

For all images, the number of classes has been optimised. Classifications have been run on the ETM image with different numbers of classes in order to assess the influence of this number on the fusion output.

The ERGAS parameter, based on an RMSE estimation (Ranchin *et al.*, 2003), is chosen as a robustness criterion (1). This statistical parameter is often used for evaluation of fusion techniques (Wehrmann *et al.*, 2005). It compares the absolute radiometric values between MeRIS original image and the one resulting from the fusion. Resolution of pixels resulting from the fusion is degraded to 300 m to be compared to the original MeRIS ones. This subsampling was performed by pixel averaging, thus considering the MeRIS Modulation Transfer Function (MTF) as perfect for the resolution decrease. This approximation has statistically no effect on the comparison.

$$\mathrm{ERGAS(X,Y)} = 100 * \frac{h}{l}\sqrt{\frac{1}{\mathrm{nb}}\sum_{\lambda=1}^{\mathrm{nb}}\frac{[RMSE(X,Y,\lambda)]^2}{[mean(X-Y,\lambda)]^2}} \quad (1)$$

with

X original MeRIS image,
Y resulting image of fusion,
h the ETM resolution (30m),
l the MeRIS resolution (300m),
λ the spectral band,
nb the total number of spectral bands.

The ERGAS is affected by two main factors. The first is the classification applied to the ETM image. It reduces the radiometric variability. The number of classes has no intrinsic physical significance. It is only a consequence of the radiometric variability within the TM image.

The second factor is the delay between the two image acquisitions. It increases the probability of landscape changes. If the classification applied to ETM is merged with a MeRIS image acquired a long time later, the fusion may become inconsistent.

4. RESULTS

A. Results on MeRIS level 1b images (see Figure 3)

Figure 2 shows that the optimal number of classes is around 100 with an ERGAS parameter equal to 1.00. Figure 3a shows a colour composite of the input ETM image (1000x1000 pixels) and Figure 3b the input MeRIS image (100x100 pixels) in radiance. A zoom factor of 10 has been applied to this image in order to emphasise the difference between ETM and MeRIS resolutions. Eventually, the resulting fusion image (Figure 3c) is characterized by 15 spectral bands and a 30 m resolution. A spatial improvement is visible when comparing Figure 3b,c.

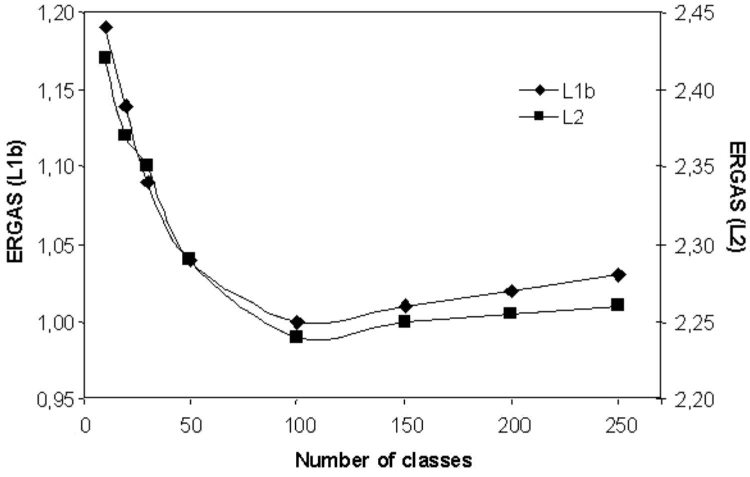

Fig. 2. ERGAS versus number of classes. The optimal number of classes is given by the minimum value of the ERGAS parameter, i.e. 100 for level 1b and 2 images.

MeRIS image spectra are well preserved, as suggested by the colour image, as well as the geometric resolution of the ETM image. The output image has the spatial characteristics of ETM and the spectral and radiometric characteristics of MeRIS (radiance image).

B. Results on MeRIS Level 2 images

The same fusion has been applied to the same MeRIS image but on the level 2 product. The optimal number of classes is also 100, and the ERGAS parameter is then equal to 2.24. This difference in ERGAS parameter values is mainly due to the artefact that MeRIS level 2 images exhibit some "black pixels" located on land-water borders.

Comparing the number of classes with the simulated case (equal to 150 (Minghelli-Roman *et al.*, 2001)), we remark that different radiometric and geomorphologic conditions provide different optimizations.

5. TEMPORAL CONSTRAINS DUE TO LANDSCAPE EVOLUTION

The revisit period of an imaging sensor depends on satellite orbit and on viewing geometry (off-nadir angle and swath width). The revisit periods of MeRIS and ETM being different and not synchronized, fusion can generally not be applied to images acquired simultaneously. Because ETM Landsat has a long revisit period (image acquisitions can occur every 16 days at minimum, and much more in cloudy conditions) and MeRIS has a wide swath (images can be acquired more frequently i.e. every three days at minimum), the fusion of ETM and MeRIS can then become inconsistent if the landscape has changed between the two acquisitions.

For example, heliosynchronous orbits are not synchronized with tide cycles, so that two images have no reason for having the same tide elevation. This may be a severe limitation in coastal areas. Similarly, seasonal landscape changes appear if the time delay exceeds a few months. The

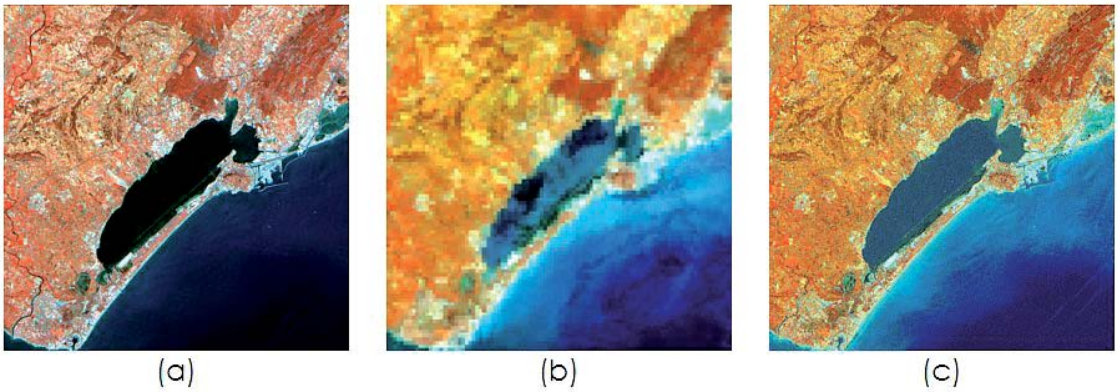

Fig. 3. (a) ETM Colour Composite (30m, 6 spectral bands); (b) MeRIS (Level 1b) Colour Composite (300m, 15 spectral bands) acquired on August 14, 2002; (c) fusion result (30m, 15 spectral bands).

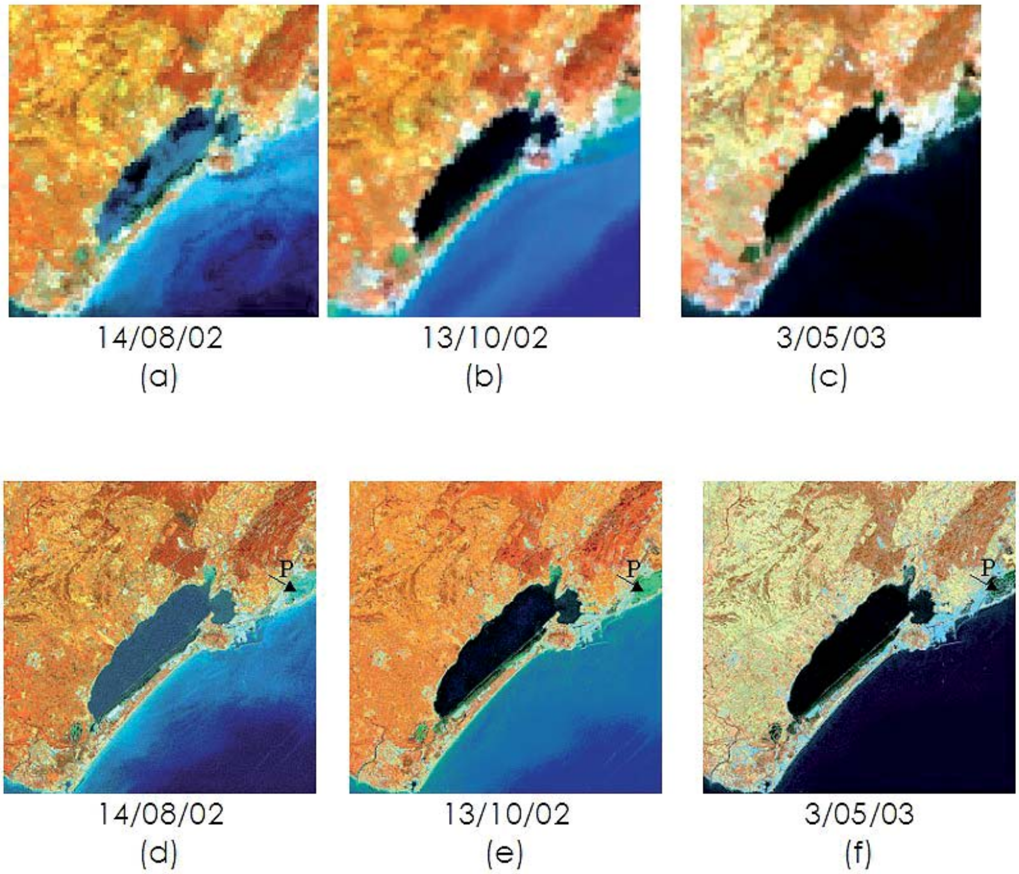

Fig. 4. MeRIS (Level 1b) colour composite images. (a)14 August, 2002; (b) 13 October, 2002; (c) 3 May, 2003; (d), (e), (f) fusion results (ETM and MeRIS level 1b) for the same dates.

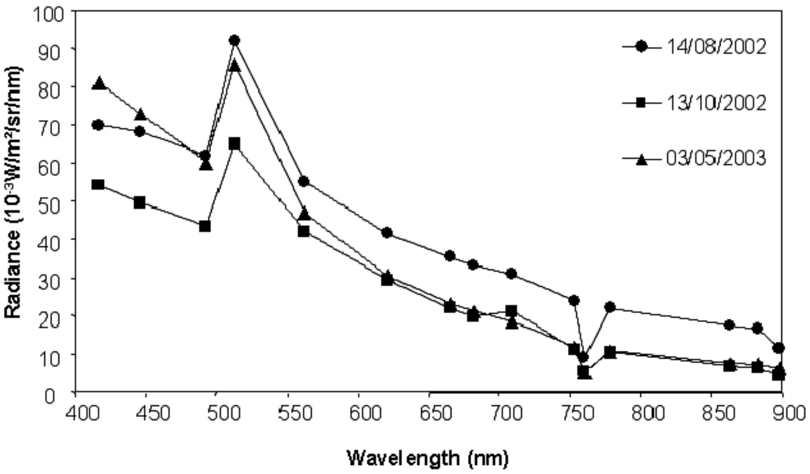

Fig. 5. Temporal spectral profile of the pixel indicated as P in Figure 4 (in radiance).

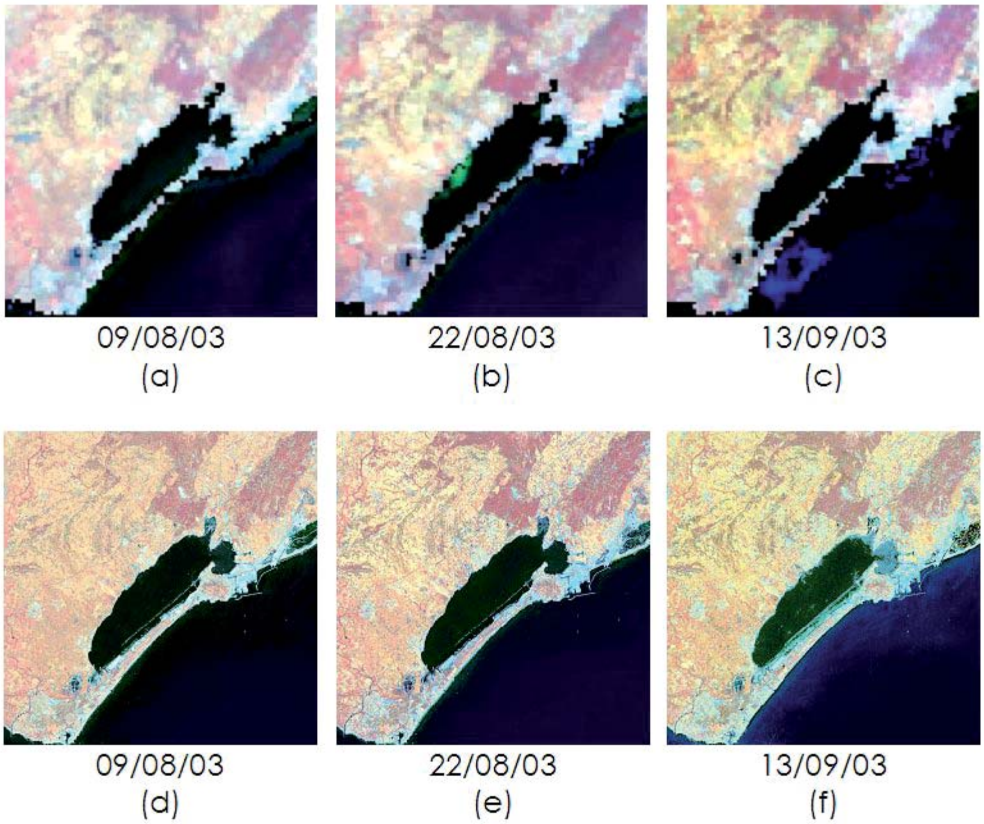

Fig. 6. MeRIS (Level 2) colour composite images. (a)9 September, 2003; (b) 22 August, 2003; (c) 13 September, 2003; (d), (e), (f) fusion results (ETM and MeRIS level 2) for the same dates.

robustness of the fusion may then be evaluated with the increasing delay between ETM and MeRIS images acquisition.

A. Results on MeRIS level 1b images (see Figure 4)

The same ETM image (1000*1000 pixels) acquired on 16 August, 2002 has been merged with three different MeRIS level 1b images acquired on 14 August, 2002, 13 October, 2002 and 3 May, 2003. The delays between ETM and MeRIS images are respectively two days, two months and nine months. All MeRIS images have been geometrically co-registered with the ETM image. Figure 4a,b,c shows these three MeRIS images (100*100 pixels).

A radiometric evolution can be observed between MeRIS images due to seasonal changes. The first image has been acquired in summer, the second one in autumn and the last in the spring. We note that inland pixels have brighter tones in the second image than in the third, because of the higher photosynthesis activity in spring.

The assumption of the geometric stability between the two acquisitions has been made. If necessary this assumption will be verified during the validation, by using the ERGAS parameter. Figure 4d,e,f shows the visual results of these fusions.

A visual comparison shows that the seasonal landscape evolutions are preserved. With the fusion, the spectral evolution of a landscape object can be tracked in the ETM image. Figure 5 shows the spectral evolution of the pixel indicated as point P, located at the middle of a pond, to the east of Thau lagoon.

Figure 5 shows a decrease of the chlorophyll peak between 14 August, 2002 and 13 October, 2002 and an increase between 13 October, 2002 and May 3, 2003. This evolution is explained by a strong eutrophication of some lagoons which receive fluvial waters containing organic matter and chemical fertilizers from cultivated fields situated upstream in watersheds. This eutrophication increases in summer under specific atmospheric conditions, decreases in autumn and winter and increases again in spring.

B. Results on MeRIS level 2 images (see Figure 6)

The same ETM image (1000*1000 pixels) acquired on 16 August, 2002 has been merged with three different MeRIS Level 2 images acquired on 9 September, 2003, 22 August, 2003 and 13 September, 2003. The image acquisition dates are different from level 1 images. The objective is not to compare the results between level 1 and level 2 images (the results would have been similar) but to show that the fusion is possible between two image levels according to the required product. The delays between ETM and MeRIS image acquisitions are respectively 358, 371 days and 393 days. MeRIS images have been geometrically co-registered on the ETM image. Figure 6a,b,c shows these three extracts of MeRIS images (100*100 pixels).

The pale colours of MeRIS images are due to atmospheric corrections which use different methods on land and water. The black pixel artefact for level 1b images is noticed on land-water borders (Figure 6a,b,c). The image of the 13 September, 2003 has many pale blue pixels which are the result of inaccurate land-water masking.

The 22 August, 2003 image shows a bright spot on the Thau lagoon, produced by an anoxic crisis. This crisis was due to the eutrophication and was resulting from specific meteorological and environmental conditions.

Figure 6d,e,f shows the fusion results for the three dates. One can notice that MeRIS radiometric values are globally preserved on water and land. Black pixels have also disappeared and have been replaced by reflectance values computed from other pixels of the same class located somewhere else in the image as explained in the method description. It is particularly noticeable on Figure 6f where the fusion has removed a important black pixel artefact.

A typical temporal effect can be seen in Figure 6e, where the lighter spot on the Thau lagoon disappeared. It is due to its absence in the original ETM image. The high reflectance of this spot has then been “diluted” in the other classes covering the Thau lagoon. This phenomenon confirms that this fusion method is only valid for a landscape with limited changes between the acquisition dates of ETM and MeRIS. Indeed, the spectral profile of a landscape object can be monitored only if it is present in the original ETM image.

Results of the ERGAS parameter calculation are given in Table 1 and Table 2.

Table 1 shows that the ERGAS parameter increases with the delay between images acquisitions. This confirms the temporal limitation of our fusion method. Ranchin *et al*. (2003) have empirically fixed a fusion validity limit for ERGAS = 3. The first three fusions with MeRIS level 1b images are valid because the landscape has not geometrically changed between the two dates.

Table 1. MeRIS Level 1: date acquisition, delay, between ETM and MeRIS acquisition, ERGAS parameter.

Image	Date	Delay between ETM and MeRIS acquisitions (days)	ERGAS
TM	16/08/02		
MeRIS level 1	14/08/02	-2	1.05
	13/10/02	58	1.33
	03/05/03	260	1.92

The fusion with MeRIS level 2 images is more critical as revealed by the ERGAS parameter which exceeds or is close to 3. Two reasons explain these high values, first the important delay between the two images acquisitions minimum 358 days, second the apparition of the lighter spot on the Thau lagoon which brought a geometrical change into the landscape. However, the ERGAS values given in Table 1 and 2 cannot be compared by absolute values because level 2 images are not obtained by a linear transformation from level 1b images.

Table 2. MeRIS Level 2: date acquisition, delay, between ETM and MeRIS acquisition, ERGAS parameter.

Image	Date	Delay between ETM and MeRIS acquisitions (days)	ERGAS
TM	16/08/02		
MeRIS level 2	09/08/03	358	3.88
	22/08/03	371	2.86
	13/09/03	393	4.66

6. DISCUSSION

The method proposed for MeRIS spatial resolution improvement has several advantages. Classical unmixing methods (Hu *et al*., 1999; Settel and Drake, 1993) require an *a priori* knowledge of end-members and their spectral profiles. This knowledge requires minimum ground information and becomes meaningless if the image does not contain pure pixels. For such methods, the number of end-members cannot exceed the number of spectral bands in order to make the system invertible and therefore to ensure that one solution exists. For the chosen fusion method, the number of end-members is only bounded by the number of available pixels in the low resolution image.

While classical methods handling absolute spectra require absolute calibration and atmospheric, directional and topographic corrections in the radiometric preprocessing, these are not required for this method.

Beyond these operational advantages, the most interesting potential of this method is the effective resolution improvement due to the fusion with a higher resolution image, while other methods only provide a proportion of each end-member inside each pixel without any subpixel location information.

However, some limitations need to be considered, some of which have already been identified (Minghelli-Roman *et al*., 2001). For example, first the merged image is only an approximation of the pseudo image acquired by a virtual 30 m resolution and 15 spectral bands instrument, and the radiometry of pixels belonging to the same class corresponds to the average radiometry measured by this virtual instrument. Second, if a landscape object was not present in the ETM image due to a drastic landscape change, it will not be present in the merged image. In both cases, the landscape evolution is more likely to occur when time delay increases between ETM and MeRIS image acquisitions, and its effects on the fusion relevance can be evaluated by comparing the ERGAS values for different time delays.

7. CONCLUSION

This paper shows how a MeRIS image can be merged with an ETM image in order to synthesize a new product with the best characteristics of each sensor, namely, the spatial resolution of ETM, and the spectral resolution and the frequent revisiting of MeRIS. The method, which had been tested on simulated MeRIS images before the launch of ENVISAT, was applied to real images over a coastal area. The effects of landscape evolution, which tend to increase as the time delay between the two acquisitions becomes longer, were analysed using the ERGAS parameter as a robustness criterion. The advantages and limitations of this resolution improvement method have been discussed. This experiment confirms the potential of this method for coastal water monitoring. However, a further validation should be carried out in areas where drastic changes are more frequent, like in tropical coastal zones.