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Health risks from water and new challenges for the future

Edited by
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Health risks from water and new challenges for the future

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Enzo Funari

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Preface

A few years ago I was invited to an international scientific meeting on water and health organized in a developing country with huge problem of water availability and quality: all sessions were opened by an authority. In my session, he complained about the state of the water in his country and felt sad that we ourselves are polluters of the same water we use for drinking, fishing, bathing, irrigating the fields. He was addressing the scientific community to ask the reasons for this paradox and possible solutions. His expression was dismayed, the testimony of a discomfort. What power of purifying polluted water can we have? How did this happen? What mistakes were made? How to fix and find the balance with the environment that surrounds us?

Undoubtedly the development of societies has not been often accompanied by the necessary capacity to protect the environment, therefore, even the water. The cause of this imbalance was due mainly to the lack of knowledge about the strict relationships between environment and health.

Currently, thanks to the numerous studies available, many of these relationships are known. Scientific knowledge has provided the basis on which many developed countries have built a modern normative, aimed at protecting the quality of the environment, including water, thus preventing people from hazardous exposures, in a context of sustainable development. The technological progress of water disinfection prior to distribution for drinking and the possibility of controlling the microbiological quality relatively easily, using bacterial indicators, have represented milestones in the history of the protection of human health.

*In developed countries, epidemics of cholera, typhoid fever belong to a distant past. However, even these countries are not exempt by problems, sometimes serious. We have discovered that bacterial indicators are not adequate to predict parasites and viruses which can be more resistant to disinfection; furthermore, results of the analysis to detect their presence are not immediate. Several outbreaks caused by emerging pathogens have been reported from national surveillance systems of developed countries (*e.g., Legionella, Cryptosporidium, *enterohaemorrhagic* E. coli, *norovirus, rotavirus, hepatitis A virus). The lesson learned from these outbreaks has guided towards new preventive approaches, such as the Water Safety Plans proposed by the WHO, where monitoring activities are only a stretch of the system necessary to ensure safe access to water.*

We also know that predicting a risk provides the possibility of preventing from dangerous exposures. A practical application of this principle is represented by the new European directive on bathing waters, where rainfalls, that can worsen the microbiological quality of these waters, are used directly as predictors of potential short-term events of contamination.

Yet, monitoring still remains an irreplaceable activity in providing information on water quality. But these activities should be tailored on the basis of the faced situation. Besides bacterial indicators specific pathogens may be added. Monitoring activities can be also enriched with innovative tools, like genotyping and biomolecular methods, particularly useful during incident and outbreak investigations in attributing sources and establishing correct interventions.

In spite of the available evidence, most of the developed countries have not set up a surveillance system on water related diseases, probably reflecting the belief that these diseases belong to the past. On the contrary, surveillance offers a systematic approach to data collection, and is crucial in helping countries to monitor and evaluate emerging patterns and trends of disease. Developed countries should strengthen national disease surveillance systems in critical areas, especially for pathologies that are currently recognized to be underreported. Data from these surveillance systems should be used to upgrade water quality management and reduce vaccine-preventable diseases, as those caused by rotavirus.

A quite recent reason of public concern is due to the awareness of the possible simultaneous occurrence in water of a wide variety of substances, as pharmaceuticals, pesticides in non agricultural areas, chemicals that interact with the endocrine system, personal care products, surfactants, nanomaterials, etc. These substances are not currently included in routine monitoring programs, hence the database on their environmental concentrations is poor. The potential adverse effects of the addivitivity and/or interactions among these substances is difficult to assess and this area represents a new scientific challenge, since the current chemicals legislation is based predominantly on assessments carried out on individual substances.

New challenges are also posed from the expansion of potentially harmful microalgae and cyanobacteria in "new" ecosystems as a consequence of intensification of intercontinental traffic, ballast waters, climate

change. This is a severe challenge from a scientific and regulatory point of view.

Recreation has a substantial role in the life of ever increasing number of citizens in the world. Man-made water recreational environments offer health promotion and social benefits accompanied with increasing comfort and sophisticated services but that can also present risky exposures to physical, microbiological, or chemical agents. In the period from 1999 to 2008 the US CDC reported 292 outbreaks attributed to "treated recreational venues", i.e. *pool, spa and similar facilities. Pathogenic protozoon with high resistance to chlorine, especially* Cryptosporidium *spp., were the most important etiological agents responsible for these outbreaks.*

Worldwide survey results show a wide variability in the absolute concentrations of natural radionuclides in waters. The highest dose fraction is often attributable to radium isotopes, that have been found only in few cases in drinking water. Yet, in some areas, radium isotopes occur at relatively high levels and specific treatments are necessary to prevent from dangerous exposures through drinking water.

Water and health is a complex issue. The risk factors that can occur in water are numerous and it is often necessary to address and possibly anticipate new ones to avoid having to recognize them in the midst of new environmental emergencies. This monographic issue is an attempt to cover some of the most relevant ones for the time being. I also hope that at least partially it contains answers to the questions raised by the religious authority in the above mentioned scientific event.

I am particularly grateful to the authors who accepted my invitation to contribute to this monographic number.

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Chemicals in the water environment. Where do the real and future threats lie?

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Abstract. There are many potential sources of chemical constituents and contaminants in water that can reach drinking water. Not all substances will be present in any particular water. Some substances may be of benefit to health but others can be a threat. However, very few have been clearly shown to cause adverse health effects in humans through drinking water and evidence may be complicated by simultaneous exposure through food. Our knowledge of contaminants in water is, however, incomplete as additional contaminants emerge with advancing analytical methods. Most of these emerging contaminants are present as a consequence of day to day use by the wider human population and control requires a different approach to the substance by substance regulation prevalent at present.

Key words: drinking water, chemicals, pollution, health.

Riassunto *(Sostanze chimiche nell'ambiente acquatico. Quali sono le minacce attuali e future?)*. Sono molte le fonti potenziali di costituenti e contaminanti chimici nell'acqua che possono raggiungere le acque potabili. Non tutte le sostanze sono presenti in ogni acqua, alcune possono giovare alla salute ma altre possono rappresentare una minaccia. Tuttavia, solo per un numero limitato di sostanze è stata chiaramente dimostrata una relazione causale per effetti avversi sulla salute associata al consumo di acqua potabile e le evidenze sono rese complicate dalla simultanea esposizione attraverso gli alimenti. La conoscenza dei contaminanti nelle acque è comunque incompleta poiché nuovi contaminanti emergono con lo sviluppo di metodi analitici avanzati. Gran parte di questi contaminanti è presente come conseguenza dell'uso quotidiano da parte di una popolazione crescente e i controlli richiedono un approccio regolatorio diverso da quello attuale basato sulle singole sostanze.

Parole chiave: acque potabili, sostanze chimiche, contaminazione, salute.

INTRODUCTION

We have discovered over time that many chemicals reach water, whether it is groundwater, rivers, lakes, estuaries or oceans. These chemicals may be highly water soluble through the whole range of solubility to virtually insoluble and they come from natural sources, from industry, human habitation and from agriculture. The sources can be point sources or diffuse sources reaching water through run-off from land or diffusion into groundwater. As our analytical capabilities have increased we can detect ever smaller amounts of chemicals and the sheer volume of information makes it difficult to determine what is important and what is not. Some chemicals, such as nutrients, have indirect effects causing blooms of algae that can have a range of impacts on aquatic ecosystems and on users of the water, or those who exploit those ecosystems. While we have learnt a great deal, our knowledge is imperfect and problems that arise are frequently localized so the issues for one water body can be very different from the issues for another.

Many chemical substances have been known to be water contaminants for many years but, for some, controls to reduce and eventually eliminate their use have been agreed and implemented, although some may be present in the environment long after we cease to use them. Some are naturally occurring and these are the ones we know do cause adverse human health effects when present at sufficiently high concentrations. Water contaminants are both inorganic and organic with the latter comprising many thousands of substances that can be possibly present in water. As indicated above the range of substances in any individual water body will vary significantly and only a small proportion of the possible contaminants will be present. Those that are may not always be present and they will usually vary in concentration at different times, sometimes significantly. When assessing the possible risks to health of water contaminants it is also important to consider exposure from other sources since drinking water is often a minor source and epidemiological studies looking just at drinking water may give highly misleading results.

Direct impacts on human health can be either through drinking water or through contamination of aquatic organisms eaten by humans. In this lat-

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ter case there is the potential for accumulation over time so that unexpectedly high amounts may be present. However, for drinking water there is often water treatment which can also vary in sophistication and effectiveness against both inorganic and organic substances. Generally the most extensive treatment will be installed on large municipal supplies taking water from surface waters that have the potential to be impacted by a wide range of pollutant sources. In addition, as water resources come under pressure from increasing demand, sources of water that would not have been considered in the past such as recycled wastewater and saline waters are being used for drinking water either directly or indirectly by augmenting traditional supplies and sources after suitable treatment.

NATURAL CONTAMINANTS

There are both inorganic and organic contaminants that arise naturally. Drinking water can contain many minerals, often in very small concentrations, that come from contact with the rocks and soils through and over which it passes. Most of these are of no concern and some, such as calcium and magnesium, may even be beneficial [1]. In addition there are substances that are essential for health, such as copper, selenium, manganese and chromium, which may also be toxic at higher intakes. For such substances any risk assessment regarding health effects must balance essentiality and possible toxicity. However, some are known to impact human health as a consequence of their presence in water. The two most important in this respect are arsenic and fluoride that are present in groundwater in many parts of the world [2]. They are a particular problem in small rural supplies for which resources are very limited and treatment may not be a practical solution. They are particularly important in areas where the water is used to irrigate rice, which takes up contaminants more than many other food crops, as shown by the accumulation of cadmium in the Toyama Prefecture in Japan which gave rise to itai itai disease [3]. Both arsenic and fluoride are significant contributors to morbidity in regions where concentrations in water are high. Arsenic causes skin and peripheral vascular disease and a variety of cancers while fluoride causes crippling bone disease [4, 5]. In the developed counties of the west they are of relatively minor importance because of the resources available to provide treatment, even at a household level for very small supplies.

There are other inorganic substances that are naturally occurring, such as selenium [6] and uranium [7], which have raised concerns. While some epidemiological studies have found both beneficial and adverse associations with exposure to selenium, which is an essential element, these studies do not take into account overall exposure and most of the adverse effects have been seen in areas where there are highly seleniferous soils and high selenium in crops. In Western Europe the greatest problem seems to be a potential for selenium intake from all sources to be too low. Uranium occurs naturally in groundwater sources in many parts of the world and the problems mainly relate to small supplies where resources are limited. Animal studies were used to derive a guideline value for drinking water by WHO but subsequent epidemiological studies, on populations exposed to much greater concentrations of uranium in water over long periods, did not provide any support for the guideline value. As a consequence the guideline value has been raised but there still remains uncertainty as to whether this is too conservative [7]. Occasional animal studies appear to show adverse effects at low concentrations but these are not consistent and the evidence from humans remains largely reassuring.

There are also naturally occurring organic contaminants that are important. Humic and fulvic acids are present as a consequence of the breakdown of plant material and are large complex molecules. Although these are not of significance on their own they are the precursors of unwanted disinfection by-products that are considered below under contaminants that arise from water treatment. Other organic substances that are directly of concern for health are toxins produced by algae in fresh and marine waters. These algae form large blooms often as a consequence of nutrients discharged into surface fresh waters and coastal waters. The key nutrients are phosphate in fresh water and nitrate in marine water. In fresh water a number of cyanobacterial species produce toxins, which include the microcystins and cylindrospermopsin that are hepatotoxins, and anatoxin A and AS that are neurotoxins. Some blooms also produce saxitoxin which is also a product of some marine dinoflagellates and causes paralytic shellfish poisoning (PSP). While the evidence of health problems from drinking water are limited to areas where blooms occur in water supplies that do not receive other than basic treatment, they can impact on livestock and pets that drink from the untreated water bodies. Although treatment can remove the toxins, preventing blooms forming is always a first line of defence [8].

The toxins which are a product of marine dinoflagellate blooms are an important cause of adverse human health effects from the consumption of shellfish that accumulate the dinoflagellate phytoplankton [9]. In many parts of the world governments have established routine screening of shell fisheries to prevent populations being exposed.

CONTAMINANTS FROM AGRICULTURE

Agriculture is one of the sources of nutrients that reach surface waters and, in the case of nitrate, groundwater that is vulnerable to leaching from the surface. The greatest concerns have been directed at nitrate contamination, particularly of groundwater, although new evidence means that there are significant uncertainties regarding potential effects. For

many years it was believed that nitrate was the main cause of methaemoglobinaemia in bottle-fed infants with concentrations of nitrate above 50 mg/litre as nitrate, the risk increasing with increasing concentration. However, increasing evidence shows that diarrhoeal disease itself significantly increases methaemoglobin formation in infants and in the absence of existing infections much higher concentrations of nitrate are required. WHO recommends that water with greater than 100 mg/litre of nitrate should not be used but that water with between 50 and 100 mg/litre can be used if it is microbiologically safe and there is increased vigilance for methaemoglobinaemia [2]. The evidence for cancer resulting from high nitrate intake through drinking water also remains equivocal at best but there is evidence that the capacity of nitrate for interfering with iodine uptake may be of greater significance. To date the evidence remains weak and certainly insufficient to identify concentrations of concern but there is a need to carry out high quality epidemiological studies to answer this question [10]. There is also increasing evidence that nitrate may have beneficial effects through the nitric oxide cycle in the body and there is endogenous formation of both nitrate and nitrite [11].

The other chemical contaminants of greatest concern with regard to agriculture are pesticides of various types. The majority of pesticides are not routinely found in water and, with the exception of spills in which the level of contamination is unusually high, the concentrations do not appear to be of significance for human health in most places, although there may be issues for some small non-municipal supplies in rural areas. In the EU, the introduction of the blanket standard for any pesticide in drinking water of 0.1 μg/litre has meant that concentrations in European drinking water are well below concentrations of concern for health. In addition there is no credible evidence for adverse health effects from pesticides through drinking water. The most toxic pesticides are of very low water solubility and so are not found at significant concentrations in water. WHO has developed conservative health-based guidelines for a number of pesticides in relation to drinking water against which observed concentrations can be compared [2]. Such guidelines are particularly useful when spills occur and drinking water is threatened over a short time as well as when there is the potential for chronic exposure since mere presence does not equate with the threat of adverse health effects if concentrations are low.

INDUSTRY AND HUMAN HABITATION

A very wide variety of chemicals may reach water from industry in industrial discharges and from the careless handling of chemicals, the most common of which are hydrocarbons from petroleum products. These latter substances are largely detected in drinking water by odour at concentrations below those of concern for health [2]. As legislation and controls on surface water contamination have been introduced, these chemicals have become less and less of an issue. In the past the discharge of heavy metals was a concern and in some developing countries it still is. However, adverse health effects from metals have not usually been directly associated with drinking water but through accumulation in crops, such as rice, *e.g.* Itai Itai disease in Japan from accumulated cadmium [3], or aquatic organisms, such as methyl mercury in aquatic life in Minimata Bay in Japan. Indeed mercury in the form of methyl mercury remains a threat to some populations that eat a mostly fish-based diet [12].

There are also concerns about highly lipophilic substances such as the polychlorinated biphenyls and polybrominated diphenyl ethers (PCBs and PBDEs) that were used in electrical equipment and as flame retardants respectively. These are controlled as persistent organic pollutants (POPs) through international conventions. They are of no consequence for treated drinking water but they have been found widely in aquatic life around the globe. In spite of the controls in place, they will be present in the environment for an extremely long time and remain a concern. While there are uncertainties regarding the extent to which they pose a threat to human health specifically through consumption of aquatic organisms minimising exposure does remain a priority [13, 14]. Unless great care is taken in finding alternative chemicals for use as fire retardants more problem chemicals will appear in the future.

More recently other persistent chemicals have emerged as potential problems for drinking water; the perfluorinated compounds such as PFOS and PFOA (perfluorooctane sulfonic acid and perfluorooctanoic acid). These substances were used as the building blocks for dirt resistant coatings on fabrics and non-stick coatings on cookware but they were also used in detergents that were widely included in fire-fighting foams, particularly for fighting aircraft fires. Although these substances are persistent in the environment, they are highly soluble and can readily reach unprotected groundwater. They are seen at elevated concentration in groundwater near manufacturing facilities but also near airports where there are practice areas for fire-fighting. Again action has now been taken to strictly control their use but the legacy will remain for some time. These substances are of concern for health but there remain uncertainties in the toxicology that are to be fully resolved. In the meantime research on exposed populations is being carried out to monitor health for a possible range of adverse effects [15, 16].

Another group of chemicals that have been frequently identified in groundwater, but not surface water, are the chlorinated solvents that were widely used for degreasing and dry cleaning in the past. Controls over handling and disposal have significantly reduced the risk of these substances polluting groundwater in the future but this may not be the case in many developing countries. Once in ground-

water they may be present for a significant period of time and some instances of contamination date back to the second world war. Other related substances are tetrachloroethene used in dry cleaning and more recently, 1,1,1-trichloroethane that was used as a less toxic replacement for the others. Where contamination has been found, particularly in the United States, there have been attempts to determine whether there have been adverse health effects through epidemiological studies. However, the concentrations are generally low and the results remain equivocal [2].

EMERGING CONTAMINANTS

With advances in analytical capability we are able to detect an increasing number of substances in water at ever lower concentrations. As a consequence our knowledge of water contaminants is increasing. These emerging contaminants will, in many cases, have been present for a long time and most are present as a consequence of domestic use, although some are quite natural, such as the hormones that are excreted by humans and animals. The primary, but not exclusive, route to water for these substances is through sewage and their presence in wastewater discharges. One group that can reach groundwater directly under areas of use is the perfluorinated compounds mentioned above but in developing countries there are cases of significant concentrations of pharmaceuticals in discharges from factories making generic compounds. While there are specific circumstances where exposure may be significantly elevated, the current discussion will relate to regions where there are controls over discharges that are enforced and where there is both wastewater treatment and drinking water treatment. The substances of interest are endocrine disruptors, classified by their potential biological activity, personal care products, which is a wide ranging group containing substances from cleaning and washing through personal hygiene products and cosmetics, and pharmaceuticals, which is another wide ranging group in terms of structures and activity.

Endocrine Disrupters are substances that are capable of mimicking or interfering with the hormonal system. Questions were first raised in the early 1980's [17] but it was when male fish living downstream from treated sewage effluent discharges were found to have ovarian elements in their testis that there was concern. It was shown that the primary cause was natural and artificial hormones excreted by humans as the glucuronides and sulphates to make them soluble. However, the conjugates are broken down in sewage treatment to re-release the parent compounds that are active and the effect rapidly disappears further downstream as the substances are adsorbed to organic material. Other chemicals also shown to have this effect were much less potent than the hormones but alkyl phenols used as detergent builders were also shown to impact fish at specific locations. Since then the alkyl phenols have been phased out. Because of the impact on fish there has been concern over the possible threat to drinking water. However, these substances are hydrophobic and are readily removed from water, which has been shown in various studies [18] and particularly by a significant study for the European Commission [19]. As a consequence the risks from drinking water where there is both sewage treatment and adequate water treatment are minimal.

Pharmaceuticals were first identified in drinking water in the early 1980's but since then there has been a significant increase in analytical capability that has shown an explosive increase in the number of pharmaceuticals and their residues detected in sources used for drinking water. All of these sources are impacted by municipal sewage effluent and the primary source in these is excretion by humans taking medication, although a small proportion comes from improper disposal of unused pharmaceuticals to sewer. There may be hot spots for discharges of specific pharmaceuticals, such as some hospitals, clinics and homes for the elderly and in developing countries uncontrolled discharges from factories making generic pharmaceuticals. A proportion of pharmaceuticals are removed in wastewater treatment and there is further degradation in surface water. Drinking water treatment will also remove a significant proportion, depending on the sophistication of the treatment and the concentration of pharmaceuticals present in the raw water. Occasionally specific pharmaceuticals may be found in groundwater from past improper disposal of pharmaceuticals by burial or to older unsealed landfill, however, this is rare, not least because of the high value of most pharmaceuticals. Several studies have examined the issues surrounding pharmaceuticals in drinking water and WHO established an expert committee which reported in 2011 [20-22]. These studies demonstrated that pharmaceuticals are present at very low concentrations, generally less than 0.1 μg/litre in water sources. Very few are found in drinking water and those that are, such as ibuprofen, naproxen, carbamazepine, benzoylecgonine and caffeine, are found at even lower concentrations, generally below 0.05 μg/litre [22, 23]. WHO has considered all of the risk assessments and has concluded that the risks to health are, at present, minimal since the concentrations present are many orders of magnitude below the lowest therapeutic doses. However, it is not possible to provide definitive reassurance for the long-term as the concentrations and numbers of substances present will change with time and there remain uncertainties regarding groups that may be particularly vulnerable, such as bottle-fed infants. In addition the question of personal care products used in toiletries and in household products such as cleaning agents remains to be

properly investigated. Since the primary source is human use and discharge through municipal wastewater treatment there is an urgent need to begin the process of improving municipal wastewater treatment to remove these and other contaminants that will emerge in the future. This treatment will need to be sustainable and not increase carbon footprint. It will also have to be introduced over a long period in view of the significant investment required and the long life of such assets [24]. This approach also means that any threat to aquatic life will also be mitigated and so must be seen as the most sustainable approach, which also fits with the Water Safety Plan approach to assuring drinking water safety introduced by WHO in the third and fourth editions of the guidelines for drinking water quality.

CHEMICALS ARISING FROM DRINKING WATER TREATMENT

While drinking water treatment and distribution is vital in protecting public health against microbial illness which remains a major cause of morbidity and mortality in many parts of the world, care has to be taken that treatment does not introduce higher levels of unwanted by-products than necessary. Such disinfection by-products (DBPs) arise from a number of sources but the best studied are those that result from the reaction between natural organic matter in water sources and chlorine used as a disinfectant. Those present at the highest concentration are the trihalomethanes that are regulated in most parts of the world and the haloacetic acids that are regulated in North America and for which guidelines have been set by WHO [2]. These molecules may also contain bromine and iodine, depending on the presence of these naturally occurring inorganic contaminants in water. There are many other chlorination by-products and they have been the subject of study over several decades since they were first discovered. However, the introduction of regulations has resulted in a significant decline in concentrations, particularly with the introduction of improved treatment to remove the natural organic matter and better filtration that means less chlorine needs to be added. Improvements in the distribution system also mean that re-chlorination as a palliative against microbial contamination in distribution is less and less common. Epidemiological studies reported weak associations between chlorination and cancers of the colon, rectum and bladder. As better exposure data were introduced, only bladder remained positive in some studies but not others. The association remains weak and currently there is no plausible mechanism for this. However, the identification of low concentrations of a range of nitrogen containing DBPs may change this, if the data can be used for epidemiological studies taking into account the changes in concentrations over the past 20 years as efforts have been made to control DBPs. WHO concluded that the data did not allow a conclusion that the association was causal [2, 25].

Several studies also reported positive associations between THM concentrations and a number of adverse reproductive effects, particularly stillbirth and low weight for gestational age [25, 26]. However, as better studies were carried out these were increasingly negative and evidence started to emerge of confounding factors. The position is now that although there is a small theoretical risk from chlorination by-products the benefits from chlorination are significant and demonstrable and chlorination continues to be practiced in most parts of the world. WHO have made it clear that although disinfection by-products should be minimized where possible, microbiological safety should never be compromised in meeting guidelines and standards for chlorination by-products [2].

Other substances arise from the treatment process, particularly aluminium where aluminium salts are used as coagulants to remove organic matter and particles, including microorganisms. Concern was expressed about the possible link between aluminium in drinking water and Alzheimer's disease following the demonstration that aluminium in dialysate was responsible for dialysis dementia in patients on kidney dialysis. Although a number of epidemiological studies reported a positive association others were negative. Further studies showed that the bioavailability from water was low and the position remained equivocal at worst. JECFA reviewed the data on aluminium from all sources and concluded that at the concentrations found in drinking water a causal association was unlikely and proposed a provisional tolerable weekly intake of 1 mg/kg of bodyweight which would translate into a drinking water value of 0.9 mg/litre assuming that 20% of the intake was allocated to drinking water, which would be conservative [27]. However, excess aluminium from treatment that is not properly optimized gives rise to the deposition of aluminium hydroxide flocs in distribution and these can cause significant problems with acceptability if disturbed. The standard of 200 μg/litre in the European Directive is quite high and larger well run treatment works should be able to achieve average concentrations of well below 100 μg/litre [2].

MATERIALS USED IN PIPEWORK

The most important contaminants from pipework used in water supply are lead and copper, which are found in the plumbing of buildings and also for connections between the public distribution system and the point at which water enters the building. Lead was used extensively for many decades as was leaded solder and a number of lead containing alloys. There are several factors that affect the dissolution of lead so that concentrations will vary

from building to building and sometimes even tap to tap. Concentrations are usually higher following extended periods during which the water is in contact with the pipes and so concentrations are often highest in first draw water in the morning but also first draw after any longer standing period. Action has been taken to reduce lead levels in drinking water as well as lead from other sources because lead is a neurotoxin and can adversely affect IQ in children and also blood pressure in adults. There is little doubt that lead in water contributed significantly to lead uptake and that there was a pressing need to reduce exposure. However, the contribution of drinking water is confounded by the contribution from other sources, particularly dust and food. The current debate is whether current low blood lead levels in children (about 2 μg/dl) are of significance and need to be further lowered. CDC in the United States has proposed altering the action level for blood lead in children to 5 μg/dl [28]. Much depends on the source but continued efforts to reduce lead exposure from sources such as old lead paint in older buildings are important. Whether lead in drinking water that meets the WHO guideline value of 10 μg/litre is a significant source of lead remains uncertain but removing lead pipes from within domestic properties is difficult and householders need to be convinced that the cost and disturbance are worthwhile. However, there are steps that can be taken to reduce lead exposure in children, such as flushing the tap and pipework after periods of standing and also removal of lead pipe and lead containing fittings in buildings where children will be exposed and where there will be frequent periods of stagnation, such as schools. These variations in exposure make determining which sources of exposure are most important very difficult and so specific research is complex, particularly since at low blood lead levels the effects will be very small and IQ is impacted by a wide range of other factors.

Copper is very different from lead and occasionally is still the cause of health effects in consumers. However, the effects are acute, reversible and relate to the concentration in the water rather than the intake over time. Copper is a gastric irritant and can cause nausea and, if concentrations are sufficiently high, vomiting [29]. Higher concentrations can occur in new copper pipe that has been left in contact with aggressive water, particularly for extended periods such as is found in new buildings or after a week-end in which the building is not used. It was also suggested that copper could be responsible for some forms of childhood cirrhosis of the liver but data no longer support that this is due to normal copper levels in drinking water [30].

DISCUSSION

Chemicals that may be present in drinking water have received increasing attention over the past 30 years, even where there is little evidence to show that they are of concern. The benefit that has accrued from this increasing awareness has been that steps have been taken to minimize the concentrations of many of these substances and particularly the few that have been shown to cause health effects through drinking water. However, as knowledge increases and as new substances may reach drinking water it is important that the potential for health effects is properly assessed and, where necessary, proper steps should be taken to mitigate any significant risks.

The substances that have been clearly shown to impact on human health through drinking water are arsenic, fluoride and, to a lesser extent nitrate and lead. It is notable that these are all inorganic substances and the major issues almost invariably relate to small supplies where the resources available are limited. Many people around the world, including Europe, receive their water from such supplies so effort needs to be directed to assuring their safety. Doubtless new potential problems will emerge and these will need to be investigated but it is important that the risks associated with chemical constituents and contaminants in drinking water are not over stated to the detriment of vigilance regarding microbiological pathogens.

Chemicals that are less of a problem for drinking water may still pose a risk to health through the consumption of contaminated staples in the diet. Metals are of particular concern in this respect through consumption of both fish and shellfish but also consumption of rice that is irrigated with contaminated water, with arsenic, cadmium and fluoride being of particular concern.

Chemicals have brought many benefits to our society and in developed countries, at least, we continue to live longer and healthier lives than ever before. While many of the environmental impacts on health relate to self-inflicted lifestyle factors, which are probably the greatest influence, chemicals that we use do have the potential to cause problems which are avoidable. The best way of dealing with these problems is prevention and to achieve this we will need to take decisions that relate to long-term change to anticipate potential future threats. To do this will require a new look at wastewater treatment and urban run-off with a view to preventing contaminants reaching the aquatic environment. Dealing with problems by a chemical by chemical approach after the event that we still tend to follow will not deliver the benefits that come from prevention of pollution.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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Long-term risks of metal contaminants in drinking water: a critical appraisal of guideline values for arsenic and vanadium

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Abstract. Metal contaminants in drinking water represent a relevant health issue in several areas of the world. In Italy, because of the geological features of the territory, high arsenic and vanadium are frequently reported in ground waters in concentrations above current guideline values. The implications for public health of the presence of contaminants above their legal limit are directly related to the biological basis of the guideline value. In the case of arsenic there are still major uncertainties in the mechanism of carcinogenesis which prevent a precise evaluation of long-term risks. Thus, the guideline value endorsed in the European Community (10 µg/L) has to be considered as a pragmatic tool rather than a quality objective, bearing in mind that "every effort should be made to keep concentrations as low as reasonably possible" (WHO, 2011). A reverse situation holds for vanadium, for which a strict national limit (50 µg/L) was previously proposed in consideration of data gaps, and for which new evidence indicated a less stringent health-based limit.

Key words: drinking water, chemical hazards, guideline values, arsenic, vanadium.

Riassunto *(Rischi a lungo termine associati alla presenza di metalli nell'acqua potabile: una disamina sugli attuali valore limite di arsenico e vanadio)*. La presenza di contaminanti inorganici nell'acqua potabile rappresenta un importante problema sanitario in varie aree del mondo. In Italia, alte concentrazioni di arsenico e vanadio, dovute alle caratteristiche geologiche del territorio, sono state segnalate in acque sotterranee usate per l'approvvigionamento idrico. Le conseguenze di ordine sanitario della presenza di contaminanti nell'acqua a concentrazioni superiori al limite legale dipendono direttamente dalle basi biologiche su cui poggia il valore stesso. Nel caso dell'arsenico, le incertezze tuttora esistenti sul meccanismo d'azione impediscono una stima precisa del rischio a basse dosi, per cui appare opportuno considerare il valore corrente come un obiettivo minimo, tenendo presente la necessità di ridurre l'esposizione umana al valore più basso realizzabile. Al contrario, nel caso del vanadio una rivalutazione sulla base di nuove evidenze scientifiche può indicare un valore limite meno stringente di quello precedentemente indicato a livello nazionale sulla base di conoscenze incomplete.

Parole chiave: acqua potabile, rischio chimico, valori guida, arsenico, vanadio.

INTRODUCTION

Drinking water quality has a heavy impact on human health. A recent assessment of major health risk factors attributed over two million deaths to unsafe water, which represents the leading environmental risk factor on a global scale [1]. The geographic distribution of mortality and burden of diseases related to water quality shows substantial differences between high-, middle- and low-income countries. Overall, more than 99% of death attributed to unsafe water occur in developing countries, where children represent the most susceptible age group [1]. The geographic and demographic patterns of risk highlight the prevailing role played by microbiological quality in determining water safety. Indeed, the presence of pathogens is still the most critical factor in determining water quality [2], despite water disinfection represented one of most significant advances of public health in last century. Chemical risk factors, however, may assume greater relevance in developed countries, where water supply is characterized by high standards of microbiological safety. Among chemical factors, regulated [2] and newly emerging [3] disinfection by-products have received major consideration as potential genotoxic and carcinogenic hazards. In addition to disinfection by-products, improvements in analytical techniques allow to identify a number of organic micropollutants (*e.g.* plasticizers, drugs, persistent organic pollutants), which represent a new challenge for the assessment and management of health risk associated with drinking water [4]. However, it is inorganic contaminants of geological origin which still raise the greatest concern, especially in selected geographical areas. In Italy, arsenic and vanadium have received major consideration

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for their biological activity and potential health impact, as well as for the frequent occurrence in groundwater at levels above their parametric values. In this paper the biological basis of the European guideline values for arsenic and vanadium in drinking water, and the implications for public health of the derogation from these limits, are briefly discussed.

ARSENIC

Basic aspects of chemistry, occurrence and biological activity of arsenic compounds, with special reference to carcinogenesis, are briefly summarized in the following subchapters. More detailed information can be retrieved from recently published reviews on arsenic exposure, toxicology and mode of action [5-7].

Chemistry, occurrence and human exposure

Arsenic (As) is a metalloid widely present in the earth's crust. The most common oxidation states are +3 (As^{+3} or arsenite) and +5 (As^{+5} or arsenate). Trivalent arsenicals are generally more toxic than pentavalent ones, due to their reactivity with sulphur containing compounds and the generation of reactive oxygen species (ROS). Both arsenic compounds can be found in inorganic and organic forms, the latter with lower or no toxicity. Concentrations of arsenic in groundwater, frequently the main source of drinking water, are usually less than 10 μg/L, but they can reach 5000 μg/L in some areas [8, 9]. Lower concentrations of arsenic are generally found in surface waters. Essentially all arsenic in drinking water is present as inorganic As (mainly As^{+5}).

Diet is the main sources of exposure to arsenic compounds for the general population, with fish and seafood, cereals and cereal products as main contributors. Recently the European Food Safety Authority (EFSA) has estimated that in Europe average consumer intake of inorganic arsenic range from 0.13 to 0.56 μg/kg b.w./day (lower and upper bound) [9]. Drinking water may represent the major contributor to dietary exposure to inorganic arsenic in areas with high natural levels of arsenic in groundwater. This is a major source of exposure worldwide, given that an estimated 160 million people live in regions with naturally elevated levels of arsenic in drinking water due to the presence of arsenic-rich geological formations and/or anthropogenic activities [2].

Metabolism and toxicokinetics

In humans inorganic arsenic is rapidly absorbed after ingestion, and subject to biotransformation which includes the reduction of pentavalent arsenate to trivalent arsenite, requiring reduced glutathione as electron donor, and the oxidative methylation of arsenite by As^{+3}-methyltransferase (As3mt) using S-adenosyl-methionine (SAM) as methyl group donor. Methylation of inorganic arsenic facilitates its excretion from the body. Arsenic toxicity in mammalian species largely depends on the rate of methylation of inorganic arsenic by liver As3mt [5], and by the rate of transport of arsenic metabolites across liver cell membrane by specific hepatic transporters. Major qualitative and quantitative inter-species differences in arsenic methylation capacity have been reported: higher activity has been observed in dog, rat and monkey compared to rabbit, mouse and humans, ascribed to the higher As3mt expression in those species [5]. Within the human species, the differential expression of As3mt associated with the AS3MT gene polymorphism plays a key role in determining the inter-individual variation in the susceptibility to arsenic induced toxicity and carcinogenicity [10].

Effects in humans

There is a strong body of evidence linking arsenic intake with a variety of health problems, from acute toxicity to chronic diseases [2]. The World Health Organization – International Agency for Research on Cancer (WHO-IARC) classifies arsenic as a known (Group 1) human carcinogen [2]. The main adverse effects reported to be associated with long term ingestion of inorganic arsenic in humans are skin lesions, cancer, developmental toxicity, neurotoxicity, cardiovascular diseases, abnormal glucose metabolism, and diabetes. Neurotoxicity is mainly reported with acute exposure from deliberate poisoning or suicide, or at high concentrations in drinking water.

Of the various sources of arsenic in the environment, long-term exposure to arsenic in drinking water is likely to pose the greatest threat to human health, and the occurrence of arsenic in drinking water has been recognized as a major public health concern in several regions of the world over the past decades [2]. Most evidence linking arsenic in drinking water with elevated cancer risk of internal organs comes from ecological studies in populations in Taiwan, Argentina, and Chile with high arsenic exposures from underground wells. Dose-related increases in the incidence of lung, urinary bladder and kidney cancers were consistently reported in population groups drinking water with arsenic concentrations above 150-200 μg/L [2, 8, 9]. At lower levels of exposure (< 100 μg/L), the available evidence is less robust and complicated by possible misclassification of study subjects, due to the difficulty in estimating past exposure, and by the limited size of most studies which make the interpretation of results more challenging [11].

Mode of action in carcinogenesis

Although the carcinogenicity of arsenic in humans has been known for more than 100 years, there is no definitive understanding of its mechanism of action for this effect. This gap in knowledge is partly due to the lack, for a number of years, of an animal model for carcinogenicity, as well as to the complex genotoxic profile and biotransformation of arsenic, and to the multiplicity of effects of arsenic compounds in biological systems.

Modes of action in arsenic-induced carcinogenicity have been extensively discussed in several recent reviews [6, 7, 12-16]. Briefly, induction of genetic damage, oxidative damage, epigenetic alterations, interference with DNA damage repair or cancer related gene proteins, have been considered as potential mechanisms, not mutually exclusive, underlying arsenic carcinogenicity. Even though the specific role of each of the proposed mechanism has not yet been disentangled, it is noteworthy that for all of them, including the induction of genetic damage, a threshold mechanism can be anticipated. As discussed later, this consideration is pivotal in risk characterization, when risk at low doses has to be extrapolated from high dose studies.

Concerning genetic damage, in particular, no direct binding or interaction of arsenic with DNA is observed. Thus, DNA damage observed *in vitro* and *in vivo* following exposure to inorganic arsenic (mainly arsenite) is attributed to indirect mechanisms such as oxidative stress mediated by increased levels of reactive oxygen species and reactive nitrogen species, and to the interference of arsenic with DNA repair and DNA damage response. The latter mechanism is also proposed to be involved in the distinct co-mutagenic and co-carcinogenic activity of arsenic [9].

Risk characterization

Several agencies have formulated quantitative estimates of cancer risk for arsenic in drinking water. Data from epidemiological studies in areas with high levels of arsenic contamination in well water were used in most cases.

The WHO/FAO Joint Expert Committee on Food Additives (JECFA) first derived a provisional maximum tolerable daily intake (PTDI) for inorganic arsenic of 2 μg/kg b.w. Based on the results from a small study in Nova Scotia, in its twenty-seventh meeting JECFA concluded that *"On the basis of the data available the Committee could arrive at only an estimate of 0.002 mg/kg b.w. as a provisional maximum tolerable daily intake for ingested inorganic arsenic."* This conclusion was based on the evidence of general toxicity (arsenicism) associated with water supplies containing arsenic concentrations ≥1 mg/L [17]. Arsenic was again considered by JECFA at its 33rd meeting, when the previous evaluation was confirmed and a provisional tolerable weekly intake (PTWI) of 15 μg/kg b.w. for inorganic arsenic established, *"with the clear understanding that the margin between the PTWI and intakes reported to have toxic effects in epidemiological studies was narrow"* [18]. The PTWI of 15 μg/kg b.w. originally set by JECFA was later criticized by the EFSA [9], and withdrawn by JECFA in 2011 [19]. In this recent JECFA opinion, data from a large prospective study in north-eastern Taiwan residents, for whom arsenic concentration in drinking water was known, were modelled to calculate the benchmark dose ($BMD_{0.5}$) associated with 0.5% increase of cancer over background. The lowest calculated BMDL0.5 value (lower 95% percentile of $BMD_{0.5}$) was 3.0 μg/kg b.w. for increased incidence of lung cancer [19]. JECFA stated that, as the new $BMDL_{0.5}$ was in the same region of the previous PTWI, this was withdrawn. It is noted, however, that BMDL and PTWI (or PTDI) have a different toxicological significance, given that BMDL is associated with a low, but not negligible, excess cancer risk.

In the United States, the Environmental Protection Agency (US EPA), based on large drinking water studies in the Taiwan population in which a dose-related increase of skin lesion was reported, using the Armitage-Doll linearized multistage model estimated for skin cancer an oral slope factor of 1.5 x 10-3 for 1 μg/kg b.w./day [20]. From this slope factor, the risk of skin cancer associated to an arsenic concentration of 10 μg/L is calculated to be 5×10^{-4} for an adult weighing 70 kg and consuming 2 L of water/day.

Other quantitative estimates of cancer risk have been formulated by the US National Research Council [21], Health Canada [22] and, more recently, by the European Food Safety Authority [9]. In its opinion on arsenic in food, EFSA modelled dose-response data from several epidemiological studies to determine the benchmark dose associated with a 1% extra risk of developing lung, bladder, and skin cancer. A range of $BMDL_1$ was identified, from 0.3 to 8 μg/kg b.w./day, the lowest value being for lung cancer. Considering the estimated dietary exposure to arsenic in Europe, calculated through an extensive survey of arsenic concentrations in food commodities, EFSA concluded that there was no or little margin of exposure and that a risk for consumer could not be excluded, and recommended that dietary exposure to inorganic arsenic be reduced [9].

Guideline value for drinking water

In Europe, quality standards for water intended for human consumption are established by the Drinking Water Directive 98/83/EC [23]. With the purpose to protect human health, the Directive sets maximum values, not to be exceeded, for a series of chemical parameters (Annex I, part B.), based on the World Health Organisation's *Guidelines for drinking water quality* and the opinion of the Commission's Scientific Advisory Committees. The guideline value for arsenic set out in Directive 98/83/EC is 10 μg/L, the same value indicated in the WHO Guidelines in 1993 [24]. Such value was derived by WHO from the previously established JECFA PTWI of 15 μg/kg b.w., allocating 20% of the PTWI to the consumption of drinking water [24].

At present, the adequacy of the guideline value indicated in the Drinking Water Directive to *"protect human health from the adverse effects of any contamination"*, as stipulated in Article 1, can be debated. In fact, even though the guideline value of 10 μg/L was reiterated by WHO in 2011 [25], a cautionary note has been introduced in the last edition of *Guidelines*

for drinking water quality. When discussing the basis for deriving the guideline value for arsenic, WHO noted that there was an overwhelming evidence of the causal relationship between consumption of elevated levels of arsenic through drinking water and the development of cancer at several sites, while there was considerable uncertainty over the mechanism of carcinogenicity and the shape of the dose-response curve at low intakes, and that the guideline value of 10 μg/L was provisionally retained *"in view of the significant uncertainties surrounding the risk assessment for arsenic carcinogenicity, the practical quantification limit in the region of 1-10 μg/litre and the practical difficulties in removing arsenic from drinking-water"*. Thus, the guideline value pragmatically indicated by WHO is not to be interpreted as a quality objective, given that, as stated in the same document, *"every effort should be made to keep concentrations [of arsenic in drinking water] as low as reasonably possible"* [25]. The latter consideration calls into question the possibility to grant temporary derogations to the guideline value, as requested – and obtained – by Italy in previous years.

Temporary derogation from the guideline values listed in Annex I can in fact be granted to Member States, provided that water supply cannot be maintained in any other reasonable way and that such derogation does not constitute a potential danger to human health (Article 9). In Italy, due to the diffuse presence of sedimentary deposits deriving from volcanic rocks, groundwater used for drinking water supply is frequently contaminated by arsenic concentrations above the guideline value. For this reason, a derogation up to 50 μg/L was allowed in 2002-2008, lowered to 20 μg/L for the period 2010-2012. Recently, following a request of the EU Directorate General for Health and Consumers, the Scientific Committee on Health and Environmental Risks (SCHER) has adopted an opinion on the potential danger to human health from the derogation on some parameters of the Drinking Water Directive 98/83/EC [26]. Concerning arsenic, the SCHER noted that recent meta-analyses of epidemiological data indicated a more than proportional decrease of cancer risk at low doses, supported by mechanistic considerations on the lack of DNA reactivity, and that no unambiguous evidence of excess risk was available for exposure at <100 μg/L. Based on these considerations, the SCHER concluded that the available information indicated that the requested derogation might only induce a very low additional tumour risk, probably less than 1/1 000 000, much less then that predicted by linear extrapolation [26].

Overall, a number of data gaps still preclude the possibility of a reliable characterization of the risk posed by arsenic in drinking water, and to set sound health-based reference values. The first data gaps concern the overall dietary exposure to inorganic arsenic, to which other food items contribute differently depending on dietary habits, and the almost complete absence of data on speciation. However, it is the lack of a comprehension of the mechanism of carcinogenicity which does not allow to develop, and apply, biologically-based models for low dose response extrapolation, providing guidance to opt between threshold or non-threshold mechanisms and to choose among the multiplicity of existing low dose extrapolation models. For the time being it can be convenient to consider the current guideline value as a pragmatic tool for risk management, and to keep in mind that at such concentration level the margin of exposure (*viz*. the distance from the effective concentration) may be small or even absent, as suggested by the EFSA [9], and that given the existing uncertainties human exposure to arsenic should be as low as reasonably achievable [25].

VANADIUM

Chemistry, occurrence and human exposure

Vanadium is a trace element widely distributed in the earth's crust at an average concentration of approximately 100 mg/kg. Vanadium exists in different oxidation states, the most common being +3, +4, and +5. Pentavalent vanadium is chemically most stable, and it represents the most toxic form [27].

Food is the main source of exposure to vanadium for the general population, with an estimated dietary intake of the order of few tens of micrograms per person per day [28]. Drinking water contributes to a lesser extent, as concentrations of vanadium in drinking water generally do not exceed few micrograms per liter. However, considerably higher concentrations (above 100 μg/L) are recorded in some water supplies, notably in groundwater from volcanic areas as consequence of the leaching from vanadium rich rocks [29].

Toxicokinetics and biological activity

The absorption rate of vanadium compounds after ingestion depends on their solubility and chemical nature. In general, however, vanadium is poorly absorbed from the gastrointestinal tract and mainly eliminated in faeces. Once absorbed, vanadium is rapidly transported by blood circulation to various tissues: the highest concentrations are initially found in kidneys, liver, and lungs, while muscles and bone represent long-term storage sites. Pentavalent vanadium predominates in extracellular fluids, whereas the tetravalent form is the most common intracellular one [28].

Vanadium in its different oxidation states is able to exert a variety of biological effects. Many of these result from the generation of reactive oxygen species during the one-electron reduction V^{+5} to V^{+4}, with subsequent DNA damage, enzyme inhibition, altered signal transduction and gene expression [30].

Toxicology

Chemical form, oxidation status, and route of exposure play a key role in determining the degree of toxicity of vanadium compounds. Orally adminis-

tered vanadium compounds (sodium and ammonium metavanadate, sodium orthovanadate, vanadyl sulphate) have been reported to produce adverse effects in kidney, spleen and lungs of rodents, to raise blood pressure in rats, and to elicit reproductive and developmental toxicity in rats and mice [31]. In humans, mild toxic effects (gastrointestinal discomfort) have been reported in subject taking high vanadium doses as food supplements [31]. Only limited oral carcinogenicity studies in rodents are available, from which no conclusion can be drawn. Similarly, no conclusion on oral carcinogenicity can be drawn from a NTP inhalational study with vanadium pentoxide [32].

A number of studies have been performed to investigate the genotoxic potential of vanadium compounds. As these studies have particular relevance for risk assessment, also in consideration of the lack of adequate carcinogenicity studies, they are briefly illustrated herein. Overall, the available results indicate that both pentavalent and tetravalent vanadium are clearly genotoxic in test systems *in vitro*, where induction of DNA strand breaks, chromosome damage and altered chromosome segregation were observed [31]. Based on current knowledge of chemistry of vanadium compounds, these effects are attributed to indirect mechanisms, such as the generation of reactive oxygen species through a Fenton-like reaction rather than to a direct interaction with DNA [31]. Consequently, the relevance of these *in vitro* findings to the *in vivo* situation is not established. The genotoxic hazard posed by the oral intake of pentavalent and tetravalent vanadium was further investigated in mouse studies specifically designed to characterize the hazard of vanadium in drinking water [33, 34]. These studies demonstrated that, following repeated administration through drinking water, only pentavalent vanadium (vanadate) is able to elicit some genotoxicity *in vivo*, and that this effect is restricted to high dose levels. Assuming a threshold mechanisms, as supported by mechanistic considerations on the genotoxicity of vanadium compounds [31], in view of the wide margin between the minimum concentrations of vanadate genotoxic *in vivo* under experimental conditions and the levels of vanadium compounds occurring in drinking water, the authors concluded that vanadium in drinking water does not raise a genotoxic concern [33, 34].

Previous evaluations

A detailed evaluation of available toxicological data on vanadium compounds was performed by the European Food Safety Authority (EFSA) with the aim of establishing a tolerable upper intake level (UL) of vanadium [31]. However, EFSA noted that the available subchronic and developmental oral toxicity studies in rats did not allow the derivation of a no-observed-adverse-effects level (NOAEL), and that no adequate evaluation of the carcinogenic potential of vanadium by oral exposure could be made. Therefore EFSA concluded that a UL could not be established, even though noted that the normal human daily intake of vanadium was at least three orders of magnitude lower than the lowest dose reported to produce adverse effects in rats [31].

A different approach was adopted overseas, where reference values were mainly based on human data. In this respect, the Agency for Toxic Substances and Disease Registry (ATSDR) derived an intermediate-duration oral MRL (minimal risk level) of 10 μg vanadium/kg/day, based on a NOAEL for hematological and blood pressure effects in humans exposed to vanadyl sulfate for 12 weeks [35]. A daily intake of 7 μg/kg b.w. was derived by the EPA as reference dose (RfD), viz. the daily exposure level without appreciable risk over a lifetime, based on gastrointestinal disturbance (intestinal cramping and diarrhoea) observed in human studies [36].

Guideline value for drinking water

As mentioned above, drinking water is not a major source of exposure to vanadium compounds worldwide. Thus, so far vanadium has not specifically been considered by WHO in its *Drinking water guidelines*, and no guideline value is indicated in the European Drinking Water Directive (98/83/EC). Yet, elevated concentrations of vanadium in drinking water have been detected in some regions, which call at least for a local regulation. In Italy, the Superior Council of Health in 1995 indicated as a limit value of vanadium in drinking water the concentration of 50 μg/L [37]. This recommendation was based on an early US EPA Health Advisory, taking into account the limitations of the toxicological database available at that time. To fill this data gap further laboratory studies, and an epidemiological survey of populations resident in areas with high vanadium in drinking water, were recommended [37].

In view of the inability to respect the 50 μg/L limit by water distribution systems serving large population groups, especially in the Etnean area in Sicily, and of the lack of evidence of overt toxicity, the limit value was provisionally raised by the Superior Council to 120 μg/L in 2000. This decision was based on a positive opinion of the Istituto Superiore di Sanità, in which however uncertainties on the genotoxic hazard of oral vanadium were noted and further experimental studies recommended. In the framework of the activities ensuing from these recommendations, the oral genotoxicity studies quoted above [33, 34] were carried out. Based on the results obtained, it can be concluded that oral vanadium does not represent a genotoxic hazard, and consequently that an acceptable human exposure level can be set using a threshold approach.

As mentioned above, EFSA failed to establish a UL for vanadium, because of the difficulty in identifying the pivotal study to be used as point of departure, and because of lack of information on carcinogenicity [31]. Concerning the latter, based on the result of the *in vivo* studies mentioned above

[33, 34], it can be ruled out that vanadium can act as a genotoxic carcinogen. Thus, no carcinogenic threat is expected for low dose vanadium in drinking water, as non-genotoxic carcinogenicity, which in principle cannot be ruled in the absence of long term studies, in any case is only elicited at high, toxic doses. Concerning the selection of the pivotal study, a critical weight was given by EFSA to early subchronic toxicity and reproduction toxicity studies, performed in one laboratory only, reporting adverse effects in rats at doses as low as 0.8 mg/kg b.w. [38, 39]. Yet, a closer view to these publications highlights severe limitations in data reporting and interpretation which casts doubts on their relevance for risk characterization. Concerning the subchronic toxicity study [38], according to the author *"... concentrations of 5, 10 and 50 ppm ($NaVO_3$) were generally well tolerated during the 3-month period … there were mild histological changes in spleen, lungs and kidneys of all the treated animals, more evident in the animals receiving the highest concentration of $NaVO_3$."*. In the absence of any other information on severity and incidence of the lesions detected, no reliable NOAEL can be derived from this study. The same group later reported a decreased body weight gain in rat pups nursed by vanadium-treated mothers [39]. Even though this finding was interpreted as a possible evidence of developmental toxicity, it can be noted that such conclusion lacks of biological plausibility, because based on the limited uptake of oral vanadium and its toxicokinetics [27], no or at most trace amounts of the element are expected in mother's milk. Thus, both studies have to be considered "not reliable" according to Klimisch criteria [40], and not to be taken into account for human risk assessment.

Despite the difficulties highlighted in the EFSA opinion, in view of the high levels of vanadium present in some groundwater serving important water supplies, and of the difficulties in removing efficiently vanadium from drinking water, there is a practical need for a biologically-based guideline value for this element. In this respect, in order to manage a few critic local situations occurring in different areas of the national territory, the Italian Ministry of Health has recently proposed a new parametric value of 140 µg/L [41], derived from a chronic toxicity study in rats receiving sodium metavanadate drinking water during a lifetime [42]. The limit value, referred to an adult weighing 60 kg and drinking 2 L water per day, was derived from a lowest observed adverse effect level (LOAEL) of 1.5 mg/kg b.w., applying a further safety factor of 3 to the 100 default value, and allocating 20 µg of vanadium to food intake. It is noteworthy that this concentration limit, derived from experiments on sodium metavanadate, the most toxic form, include all vanadium compounds, including the least toxic as tetravalent vanadium. This adds a further margin to the default safety factors incorporated in human risk assessment. This additional factor, related to vanadium speciation, is particularly relevant for drinking water, where only a fraction of total vanadium is in the highest oxidation status [27]. It is advisable that this, or another common guideline value, be soon endorsed at the Community level.

CONCLUSIONS

Metal contaminants in drinking water still represent a relevant health issue in several areas of the world. In particular, the presence of high arsenic and vanadium in groundwater, usually linked to the geological characteristics of the territory, is a challenging task for risk managers, especially when alternative sources of water for human consumption are not available. In these circumstances the definition of sound, biologically-based guideline values acquires major relevance, as guideline values may represent the key tool for the efficient management of environmental threats, in principle allowing the best protection of human health with the minimum waste of material resources. As discussed in this report, at present internationally agreed guideline values for arsenic and vanadium in drinking water are not available, or subject to considerable debate. A re-assessment of the existing limit values, or the definition of new ones, based on state-of-the art science can thus be considered a priority issue in the environmental health agenda.

Conflict of interest statement

No financial interest is declared. The authors were co-authors of two of the publications quoted [33, 34], performed within a scientific cooperation between Istituto Superiore di Sanità and Azienda Consortile Servizi Etnei (A.CO.S.ET) in the framework of an *ad hoc* action plane coordinated by the Ministry of Health (2000-2004) .

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Radioactivity in drinking water: regulations, monitoring results and radiation protection issues

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Abstract. *Introduction.* Drinking waters usually contain several natural radionuclides: tritium, radon, radium, uranium isotopes, etc. Their concentrations vary widely since they depend on the nature of the aquifer, namely, the prevailing lithology and whether there is air in it or not. *Aims.* In this work a broad overview of the radioactivity in drinking water is presented: national and international regulations, for limiting the presence of radioactivity in waters intended for human consumption; results of extensive campaigns for monitoring radioactivity in drinking waters, including mineral bottled waters, carried out throughout the world in recent years; a draft of guidelines for the planning of campaigns to measure radioactivity in drinking water proposed by the Environmental Protection Agency (ARPA) of Lombardia.

Key words: radioactivity, drinking water, water quality, public health.

Riassunto *(Radioattività nell'acqua potabile: le normative, i risultati dei monitoraggi e i problemi di radioprotezione). Introduzione.* L'acqua potabile contiene normalmente molti radionuclidi naturali: trizio, radon, isotopi del radio e dell'uranio, ecc. La loro concentrazione è molto variabile perché dipende dalla natura dell'acquifero, dalla presenza di aria in esso e dalla litologia prevalente. *Scopi.* In questo lavoro è presentata un'ampia rassegna sul tema della radioattività nelle acque potabili: le norme nazionali e internazionali per limitare la presenza di radioattività nell'acqua destinata al consumo umano; i risultati di estese campagne di monitoraggio nelle acque potabili, comprese le acque minerali imbottigliate, condotte nel mondo negli ultimi anni; una proposta di linea guida per la pianificazione di campagne di misura della radioattività nell'acqua potabile elaborata dall'Agenzia Regionale di Protezione Ambientale (ARPA) della Lombardia.

Parole chiave: radioattività, acqua potabile, qualità dell'acqua, sanità pubblica.

INTRODUCTION

Radionuclides of natural origin are normally present in different amounts in drinking water. They are released from rocks and minerals which form the aquifer as happens with other cations and anions: processes of erosion and dissolution bring radioactive elements from rocks into the water [1]. Some common natural radioelements are those from the uranium-238 chain, a natural radioactive series of many radionuclides, one descending from the other. The most relevant are uranium-238 (^{238}U), uranium-234 (^{234}U), radium-226 (^{226}Ra) and radon-222 (^{222}Rn). Relatively abundant in the earth crust, ^{238}U (around 3 mg/kg) and its descendant ^{234}U, are often the most abundant radionuclides in water. The occurrence of (^{222}Rn) may be important too; indeed, because of its moderate solubility in water, it rises mostly by emanation from inner soil layers rather than from the decay of the dissolved parent (^{226}Ra). In spite of the fact that the activity concentration of ^{222}Rn in freshly drawn water may be more than two orders higher than that of other radionuclides, it readily diminishes as a result of both desorption and physical decay.

Another crucial radioactive family is the thorium-232 series. Thorium is 3-4 times more abundant than uranium in the earth's crust, but owing to its poor solubility it scarcely occurs in waters. Radium-228 (^{228}Ra) belongs into this series and it may become a critical contaminant, as its radiotoxicity is relatively high.

Potassium-40 (^{40}K or K-40) is also a widespread radionuclide. It is a beta-gamma emitter of primordial origin and goes along stable potassium in a fixed ratio (31.3 Bq per gram of stable potassium).

Tritium (^{3}H, H-3 or T) is a cosmogenic radionuclide; it is a hydrogen isotope formed in the high atmosphere. It occurs in rainfalls as tritiated water (HTO) at a concentration of roughly 5 Bq/l. It can be also produced by anthropogenic activities (research and nuclear facilities). It can reach the aquifers along their recharge process, but its concentration progres-

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sively decreases due to its relatively short decay time ($t_{1/2}$ = 13 years). Tritium has a low radiotoxicity. In fact its dose coefficient is three orders of magnitude lower than the coefficients of the natural chain radionuclides [2].

Besides natural radioactivity, exposed water reservoirs can be contaminated by artificial radionuclides (*i.e.* rivers and lakes by radioactive fallout caused by accidents or nuclear explosions) and for these reasons, allowed levels of artificial radionuclides in case of emergency have been set. A recent example is drinking waters in Tokyo during the Fukushima accident [3]. In routine situations, reference levels – chosen to give a non-significant dose to the population – are set much lower than emergency levels.

There is also contamination by natural radionuclides caused by human activity, where those radionuclides have been concentrated by non-nuclear industrial processes (*e.g.* mining, coal combustion, fertilizer production, etc.).

BASICS OF NATURAL RADIOACTIVITY TRANSFER TO WATER

In the rock lattice all elements belonging to the same radioactive series are in secular equilibrium (their activity is the same). However, the radionuclide sorption from the rock and the following stability as solutes is ruled by complex chemical-physical mechanisms and by the individual characteristics of radioelements. Thus, the secular equilibrium is lost for dissolved radionuclides and their concentrations in water are rather independent one from each other. Some general behaviour rules for radioactive elements are given here, mainly gathered from extensive studies run in the US [1, 4]:

- radionuclides produced by alpha decay are more readily driven out from rock: alpha decay causes the atom to recoil, which reduces atom stability in the lattice (*i.e.* ^{234}U activity concentration in water is higher than, or equal to, that of the parent ^{238}U because alpha decay-induced recoil can expel ^{234}U from rock);
- highly porous rocks (*e.g.* basaltic rocks) can easily reabsorb radionuclides from water. On the opposite, sandstones have a very low capability of absorption;
- radium has a relatively low solubility and does not form soluble complexes. Usually uranium isotopes (^{234}U and ^{238}U) are the most abundant radionuclides in water;
- in oxidizing conditions, uranium forms soluble stable complexes (*e.g.* carbonates) and can move for long distances;
- in reducing conditions (absence of air) uranium precipitates, forming concentrated secondary deposits. Levels of the descendant ^{226}Ra can be very high in sites of uranium enrichment;
- being its solubility very low, thorium is scarcely mobilized by water. The transport of the descendant ^{228}Ra is limited by its short half-life (5.7 years). This means that levels of ^{228}Ra are directly controlled by the concentration of thorium in aquifer solids. Owing to the higher abundance of Th over U, ^{228}Ra can be the dominant radium isotope if there is no secondary enrichment of uranium (secondary enrichment is defined as the sum of precipitations and re-dissolutions phenomena which occur in different zones of the aquifer);
- highest values of ^{228}Ra can be found in waters originated from granitic rocks, arkosic sand and sandstones (geometric mean 50-80 mBq/l, 95° percentile 450-650 mBq/l), quartzose sandstone aquifers. No relevant changes are recorded in radium concentrations over time;
- the dependence of ^{226}Ra on the local geology is not so sharp due to the higher mobility of the radionuclide and of the parent ^{238}U;
- in roughly geologically homogeneous areas, a linear dependence between radium isotope concentration and total dissolved solids can often be found;
- radionuclide concentration is higher in ground water than in surface water.

NATIONAL AND INTERNATIONAL REGULATIONS

Many national and international institutions have published rules – mandatory or not – that carry the intent of managing the question of radioactivity in drinking water. A short review of the most significant ones is hereby presented.

World Health Organization (WHO)

WHO guidelines for drinking water suggest performing an indirect evaluation of individual dose criterion (IDC) of 0.1 mSv/y by measuring gross alpha and beta radioactivity and checking compliance of radionuclide activity concentration to derived guidance levels [2].

Once an IDC of 0.1 mSv from 1 year's consumption of drinking water has been adopted, the recommended assessment methodology for controlling radionuclide health risks from drinking water involves three steps [2]:

- initial screening is undertaken for both gross alpha activity and gross beta activity. If the measured activity concentrations are below the screening levels of 0.5 Bq/l for gross alpha activity and 1 Bq/l for gross beta activity, no further action is required;
- if either of the screening levels is exceeded, the concentrations of individual radionuclides should be determined and compared with the guidance levels (see Table 9.2 in [2]);
- the outcome of this further evaluation may indicate that no action is required or that further evaluation is necessary before a decision can be made on the need for measures to reduce the dose.

Table 1 | *Reference values for radioactivity concentration in drinking water in the current EU legislation*

Regulations	Reference values		
EU, 1998 [5]	Tritium: 100 Bq/l	TID:0.1 mSv/y	
Euratom, 2001 [8]	Rn-222: 100 Bq/l	Po-210: 0.1 Bq/l	Pb-210: 0.2 Bq/l

A specific guidance level is indicated for uranium. In fact, "The provisional guidance value for total content of uranium in drinking water is 30 µg/l based on its chemical toxicity, which is predominant compared with its radiological toxicity" [2].

European Union

Non-emergency situations

Concern about the total content of radionuclides in drinking water was brought to public attention by Council Directive 98/83/EC on the quality of water intended for human consumption. The directive requires Member States to monitor the concentrations of radionuclides in public drinking water [5] and sets parametric values of 100 Bq/l and 0.1 mSv/y for tritium activity concentration and total indicative dose (TID), respectively. The Directive sets out that TID must be evaluated excluding tritium, ^{40}K, ^{14}C, (^{222}Rn) and its decay products, but including all other natural series radionuclides. The Directive also reports an important note (Note 10 of radioactivity Table in [2]): "A Member State is not required to monitor drinking water for tritium or radioactivity to establish total indicative dose where it is satisfied that, on the basis of other monitoring carried out, the levels of tritium and of the calculated total indicative dose are well below the parametric value. In that case, it shall communicate the grounds for its decision to the Commission, including the results of this other monitoring carried out". For example, when it is well known that a possible source of tritium is not present in the area of interest, monitoring of this radionuclide is not needed.

In 2001 this Directive was transposed into national law in Italy [6]. It is important to point out that neither Directive 98/83/EC nor the Legislative Decree (DL.vo 31/01) which transposes it in Italy apply to all bottled waters other than mineral waters. The increasing use of mineral waters can pose radiation protection problems as they are generally richer in natural radioactivity than tap water is, but many people perceive and consume them as if they were tap water (*e.g.* to prepare baby/infant formula milk). For this reason mineral waters have been monitored in some Italian Regions (see par. on monitoring campaign).

Tritium determination follows a well-established procedure, standardized by the International Standard Organization [7]. Conversely, total indicative dose evaluation requires more specific and cumbersome procedures for the measurement of the radioactivity content, with special regard to natural series radionuclides. The large number of possibly involved radionuclides and the high sensitivities required make the application of traditional analytical techniques unsuitable for large scale monitoring programs.

Maximum concentration values for radon and its decay products, out of the application field of the directive, were separately proposed in Commission Recommendation 2001/928/Euratom [8]. It is worthwhile remembering that the reference values for ^{222}Rn and its short life decay products are not mandatory.

Table 1 shows a summary of the European regulations.

On 17 April 2012, a new Council Directive "laying down requirements for the protection of the health of the general public with regard to radioactive substances in water intended for human consumption" was submitted to the European Parliament for final approval [9]. This new regulation introduces important novelties with respect to Directive 98/83/EC [5], namely; 1) it regulates Rn and its decay products; 2) it reports general principles and monitoring frequencies for water, and screening levels for gross alpha and beta activities; 3) it explains the method of TID calculation; 4) it provides a list of the activity concentrations of the most common natural and artificial radionuclides to comply with a TID of 0.1 mSv per year; 5) for said radionuclides, it provides requirements on detection limits of the analytical methods utilised.

Accidents and emergency situations

After the Chernobyl accident, the European Union set maximum permitted levels of radioactive contamination of foodstuffs in case of a radiological emergency. Maximum reference levels were proposed separately for alpha-emitting artificial radionuclides and beta- and/or gamma-emitting artificial radionuclides in drinking water [10]. These values will be in force in any case of an emergency situation *(Table 2)*.

Table 2 | *Maximum permitted levels for liquid foodstuffs (Bq/k) [10]*

	Liquid foodstuffs* (Bq/kg)
Isotopes of strontium, notably Sr-90	125
Isotopes of iodine, notably I-131	500
Alpha-emitting isotopes of Pu and trans-plutonium elements, notably Pu-239 and Am-241	20
All other nuclides of T1/2 >10 days, notably Cs-134 and Cs-137	1000

**Values are calculated taking into account consumption of tap-water and the same values should be applied to drinking water supplies at the discretion of competent authorities in Member States.*

Table 3 | *Maximum levels (Bq/kg) for liquid foodstuffs as provided in different phases by the Japanese legislation and adopted by the European Commission [12, 13]*

	Liquid foodstuffs (from 25/03/11 to 31/03/12)	Mineral water and similar drinks and tea brewed from unfermented leaves (from 01/04/2012)
Sum of Isotopes of strontium, notably ^{90}Sr	125	
Sum of Isotopes of iodine, notably ^{131}I	300	
Sum of alpha-emitting isotopes of plutonium and trans-plutonium elements, notably ^{239}Pu, ^{241}Am	1	
Sum of all other nuclides of half-life > 10 days, notably ^{134}Cs and ^{137}Cs	200	10

Special legislations have been issued to regulate import from Japan in the aftermath of the Fukushima accident. These official initiatives of the European Commission [11, 12] were necessary to align the EU levels with the significantly lower Japanese ones, estimated to determine 5 mSv/y (1 mSv/y from drinking water). One year after the accident, Japanese authorities reduced the dose goal to 1 mSv/y (0.2 mSv/y from drinking water); consequently, the European Commission issued a new regulation [13]. *Table 3* reports levels for liquid foodstuffs.

Some remarks are necessary here. First of all, the cited regulations – issued after the Fukushima accident – only concern import from Japan [11-13]. This means that in case of an emergency situation coming from a EU or non-EU country, Regulation 3954/87 [10] is automatically enforced *(see Table 2)*. Secondly, the total amount of Japanese liquid foodstuffs to be assimilated to drinking water and imported into the European Union is actually small (around 2% of food imports); which means that the adoption of levels well below those which would be applied in EU in case of radiological emergency does not determine a significant problem of import and marketing. Finally, from a radiation protection point of view, in the authors' opinion, Japan's conservative maximum levels should kindle the revision of the EU emergency maximum levels in foodstuffs.

US Environmental Protection Agency (USEPA)

In 2000 the US Environmental Protection Agency [14] published the final rule about radionuclides in drinking water. In this regulation, maximum contaminant level goals (MCLGs), maximum contaminant levels (MCLs), and monitoring, reporting, and public notification requirements for radionuclides are given. The rule is only applicable to community water systems. MCLGs (non-enforceable health-based targets) are zero for all radionuclides, based on the no-threshold cancer risk model for ionizing radiation. The other requirements are summarised in *Table 4*.

The regulation also reports requirements for the detection limits of all radionuclides, and indicates the best measurement techniques *(*see *Table 5)*.

Table 4 | *USEPA maximum contaminant levels (MCLs) for radionuclides in drinking water (other than radon) [14]*

Contaminant	MCL
Combined radium-226 and radium-228	5 pCi/l (0.185 Bq/l)
Gross alpha (excluding Rn and U but including Ra-226)	15 pCi/l (0.555 Bq/l)
Beta particle and photon radioactivity	4 mrem/year (0.04 mSv/year)
Uranium	30 µg/l

AVAILABLE RESULTS OF MONITORING CAMPAIGNS

From the beginning of the '80s, wide monitoring campaigns for assessing the natural radioactivity in drinking waters have been run in United States [15-18]. Results have been summarized by J. Longtin [4]. Mostly uranium isotopes, ^{226}Ra, ^{228}Ra, and ^{222}Rn were measured.

Subsequently extensive studies have been performed both on ground waters feeding public water resources [19-22], and on spring or bottled mineral waters [23-31]. Many other studies are available, including reports by national authorities [32-34].

In Italy the first papers on radioactivity in waters were published starting from the '60s [35, 36]. One or

Table 5 | *USEPA required regulatory detection limits for the various radiochemical contaminants [14]*

Contaminant	Detection limit pCi/l (Bq/l)
Gross alpha	3 (~ 0.110)
Gross beta	4 (~ 0.150)
Radium-226	1 (~ 0.040)
Radium-228	1 (~ 0.040)
Cesium-134	10 (~ 0.400)
Strontium-89	10 (~ 0.400)
Strontium-90	2 (~ 0.075)
Iodine-131	1 (~ 0.040)
Tritium	1 000 (~ 40)
Other radionuclides and photon/gamma emitters	1/10th of the rule

two decades later extensive studies were done mainly by the Milano University team [37-44]. Afterwards, public agencies for environmental monitoring started the surveillance of water resources [45-47]. In the meantime systematic campaigns for assessing levels of radon in waters started as well [48]. Since 1996 the Environmental Protection Agency of Lombardia (ARPA Lombardia) has performed detailed studies on radioactivity in both mineral and tap waters [49-55]. Radiometric data on drinking waters are available for central Italy too [56-61].

Worldwide survey results show a wide variability in the absolute concentrations of natural radionuclides (which usually follow a lognormal distribution). If freshly drawn tap water is considered, the highest radioactivity concentration is generally due to ^{222}Rn, which is 2-3 orders higher than for other radionuclides. Nevertheless, due to both ease of desorption and physical decay, its concentration rapidly decreases and is significantly lower if sampling is made at the delivery point (in-house water tap) or negligible in bottled water. If the total exposure due to drinking water is calculated, ^{222}Rn can be generally responsible of the largest dose fraction, mainly by the inhalation pathway [34].

Among non-volatile radionuclides (thus excluding ^{222}Rn), the uranium isotopes often show the highest activity concentrations but, given their relatively low conversion factor, their contribution to the total dose is low [62]. The highest dose fraction is often attributable to radium isotopes (^{226}Ra and ^{228}Ra), especially when lower age classes are taken into account. In some cases the major contribution is due to ^{210}Pb and ^{210}Po [33, 34].

Crucial for radioprotection, the problem of radium contamination has been widely examined. The presence of radium isotopes in groundwater is highly variable; the concentrations of ^{226}Ra, and sometimes those of ^{228}Ra, may exceed 1 Bq/l, but in most cases they are associated with thermal springs, mineral water or drilled wells. Very few examples are found of aqueducts contaminated by radium [63]. In Finland many investigations have been carried out even in recent times [32, 64]. The problem of radium concerns only private wells and to a limited extent: 4% of the examined wells exceeded 500 mBq/l of ^{226}Ra and 1% exceeded 200 mBq/l of ^{228}Ra concentration. In Sweden [65] a considerable number of private drilled wells (47%) exceeds the national limit (185 mBq/l for total radium). Measurements carried out in Western Spain [66] showed that in 13% of drilled wells ^{226}Ra exceeded 1 Bq/l, with a maximum value of 9.3 Bq/l, but dug wells exhibited much lower radium levels. In Extremadura, close to Portugal, small aqueducts carry water with ^{226}Ra content up to 720 mBq/l and mitigation processes have been considered [67]. Radium monitoring in water resources has been accomplished in many other countries [33, 51, 68-74]. Interesting cases are found in Middle East. In some dry areas in the south of Israel [75], the wide Nubian sandstone aquifer produces water with radium activities exceeding national regulation limits, which are in substantial agreement with WHO screening criteria. In any case, in such dry areas there are few alternative resources. In southern Jordan, nearby the cited area, the possibility of exploitation of this fossil, non-renewable aquifer has been studied [76]. Concentrations up to 1.3 Bq/l and 3.1 Bq/l for ^{226}Ra and ^{228}Ra, respectively, have been found with highly variable ratios.

As for the USA, nearly 80% of the 60 000 water supplies in the country use ground water sources, and 90% of them serve less than 3300 people. In general radium was found to be a problem for some small aqueducts. A screening level of 185 mBq/l for gross alpha activity and 110 mBq/l for ^{226}Ra was used: non-compliances were found mainly in South-East coastal regions (New Jersey, North Carolina, South Carolina, Georgia) and in the North-Central regions (Minnesota, Iowa, Illinois, Missouri and Wisconsin) [4]. Those non-compliances were mainly due to ^{226}Ra and the average radium isotope concentration was 370 mBq/l. In South Carolina approximately 3% of the ground water supplies exceeded the 185 mBq/l value, reaching the values of 980 and 440 mBq/l for ^{226}Ra and ^{228}Ra, respectively. In Iowa radium concentrations up to 1.8 Bq/l (before treatment) were reported for a public water supply [63, 77]. As a consequence, a number of aqueducts adopted specific treatments for radium mitigation in drinking waters.

If we consider the above literature data, radium levels comparable with derived levels (^{226}Ra > 500 mBq/l or ^{228}Ra > 200 mBq/l) [62] are seldom found in public water resources. Most cases are related to private drilled wells (especially in Scandinavia) and to mineral or thermal water, whose consumption is optional. In some areas (*e.g.* Australia) local uranium mining may cause water contamination but it does not concern drinking water resources. Aqueducts using ground water normally exhibit low radium levels even in areas whose prevailing geology is supposed to be favourable to the radiological contamination of waters. Situations in which radium levels are high and water treatment is necessary for Ra removal are spatially limited exceptions. One remarkable example is Estonia, where aqueducts in the North are fed by aquifers very rich in radium isotopes [78-80]. This gave rise to a high number of non-compliances with the national regulation, since Estonia national law adopted a TID value of 0.1 mSv/year [5] as a limit. The highest radium concentrations are known to be found in the oldest and deepest Estonian aquifer [81], the Cambrian-Vendian (Cm-V). This aquifer becomes shallower in northern Estonia, close to the coastal area, where it is widely used as a drinking water reservoir. Being the most populated area as well, the radioprotection concern involves a high percentage (22%) of the Estonian population, roughly estimated in 250 000 people [82]. This recent study shows that doses to the younger population often exceed the value of 1 mSv/year in, up to 12

mSv/year. The potential impact on human health drove the European Commission to establish a cooperation agreement between Italy and Estonia in order to better understand the situation and implement remedial actions [82].

GUIDELINES DRAFT

While waiting for the new, more detailed EU directive [9], in the Annex a draft of guidelines for the planning of campaigns to measure radioactivity in drinking water proposed by ARPA Lombardia (Environmental Protection Agency of Lombardia) [83] is presented. The document was commissioned by ISPRA (National Institute for Environmental Protection and Research) in order compensate for the lack of measurement methodologies and protocols in DL.vo 31/01 and while waiting for the more detailed, new EU directive [9], and was prepared by ARPA Lombardia with the contribution, among others, of ARPA Emilia Romagna and the Italian National Institute of Health (Istituto Superiore di Sanità, ISS). The draft was presented in 2005 during the Annual Meeting of the Italian Network of Environmental Radioactivity Monitoring, but was never officially released. In these guidelines some indications for starting a survey are presented, which account for approaches already adopted by some international organizations [2] and discussions carried out in the European context [84].

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Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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ANNEX

Guideline draft for the planning of campaigns of measurement of radioactivity in drinking water [83]

Legislative Decree 31/01 (DL.vo 31/01) "Transposition of Directive 98/83/EC on water intended for human consumption" [6] for the first time set the obligation to verify the content of natural and artificial radioactivity in water. The recommendation of the European Communities 2001/928/Euratom [8] considers the problem of population exposure to radon-222 and some of its decay products (lead-210 and polonium-210) in drinking water, and aims to promote the implementation of representative surveys to establish the extent and nature of exposure to radon and its long-life decay products.

The control of water is to be performed by both the waterworks (internal controls) and the local health authority (external controls). As for radiometric parameters, control planning should be defined by Regions, in accordance with the requirements of annex II of the DL.vo 31/01. However, neither Directive 98/83/EC [5] nor the DL.vo 31/01 [6] have yet defined frequency, methods or criteria for the choice of control points. In any case, Italy has felt the need to undertake these controls, to be carried out by the Regional Environmental Protection Agencies (ARPA). For lack of specific guidelines, a useful tool can be the European Commission recommendation 2000/473/Euratom on the control of environmental radioactivity [85] – which provides general guidance on planning criteria for environmental monitoring.

At any rate, after some ARPAs first advanced the 2001 proposal to define ways of planning and conducting investigations on radioactivity in water intended for human consumption [86], this guideline intends to provide methods and criteria to plan measurements with specific reference to the parameters and values set out in Legislative Decree 31/01. Radon-222 measurement is not an explicit goal of this document, as this type of assessment is required neither by Legislative Decree 31/01 nor recommendation 2000/473/Euratom.

Legislative Decree 31/01 (DL.vo 31/01)

As regards radioactivity in drinking water, DL.vo 31/01 requires the compliance with the limits of two parameters, *i.e.*, the concentration of tritium (H-3) and the value of the total indicative dose, TID *(Table A1)*.

Tritium is a radionuclide of both natural and artificial origin. As already said, tritium is produced by the interaction of cosmic radiation with the upper atmosphere, enters the water cycle and is normally found in drinking water at concentrations of about some Bq/l. Sources of anthropogenic tritium are some types of research and nuclear facilities.

TID depends on the amount of radiation absorbed by the body after the ingestion of radioactive substances contained in water; it is measured in mSv/year. The dose from ingestion is normally estimated by multiplying the values of radioactivity concentration in water by specific conversion coefficients that depend on the type of radioactive substance. Dose assessment requires the measurement of all the radioactive isotopes present in the water, excluding the contribution of tritium, potassium-40, radon-222 and its decay products. This method of investigation, however, is extremely costly because it requires a great amount of time and resources (the amounts of radioactivity to be searched for are very small, which requires the use of very sensitive analytical techniques). It is therefore not applicable to a large number of samples.

In order to compensate for the lack of measurement methodologies and protocols in DL.vo 31/01 − and while waiting for the more detailed, new EU directive [9] −, some indications for starting a survey are presented, which account for approaches already adopted by some international organizations [2] and discussions held within the European context [84].

The Italian scenario

In Italy numerous studies on the content of radon-222 in water, explicitly excluded from the scope of the DL.vo 31/01 [6], indicate that the concentration of this radionuclide is always less than 100 Bq/l [38, 47, 86, 87], with some exceptions in the pre-alpine and alpine areas.

Table A1 | *Parameter values established by the DL.vo 31/01 for radioactivity [6]*

Parameters	Parameter value that must be observed
H-3	100 Bq/l
Total Indicative Dose *	0.10 mSv/year

**Contribution of tritium, K-40, radon and radon decay products should not be taken into account.*

For some years now, periodic assessments of the artificial radionuclide contamination of drinking water are in place in several Regions. Unfortunately, such data are not sufficient to assess the parameters required by the DL.vo 31/01 because they are acquired by gamma spectrometry, a method which does not measure pure alpha- or beta-emitting radionuclides − in particular uranium and radioisotopes −, and often shows insufficient analytical sensitivity. Surveys of radioactivity in waters, valid also for DL.vo 31/01 requirements, were carried out in Lombardy [53]. Sporadic measurements of tritium were conducted in other Regions.

Proposals for planning surveys under Legislative Decree 31/01

Control surveys of radioactivity in water should be planned, accounting for the fact that the levels of radioactivity to be measured are very low: some mBq/l or fractions of mBq/l of strontium-90 and cesium-137, some Bq/l of tritium, from few mBq/l to a few hundred mBq/l for gross alpha and beta activity, and radium, uranium and thorium isotopes. Analytical methods must thus be higly sensitive, and turn out to be onerous in terms of time and human efforts. This is why radioactive control plans cannot match plans already in place to evaluate chemical and microbiological parameters, which surely have a higher number of samples per year. Moreover, since the most substantial contribution to total radioactivity in water is of natural origin (except in the case of accident or in the presence of important anthropogenic sources) [14, 88], the radioactivity content in water is unlikely to change significantly over time. The frequency of inspections, therefore, does not necessarily have to follow DL.vo 31/01 requirements for non-radiometric parameters.

Furthermore, as already proposed in some international documents [2] and during discussions at the European level, a procedure to perform an initial screening of the gross alpha and beta activity concentration is hereby presented. This method is less complex and less costly than single radionuclide measurements, can be carried out on a large number of samples, and provides useful indications on the total radioactivity content of the water. The suggestions provided in this document about application times and procedures for measurement surveys are provisional, in waiting for the new European Union Directive [9] and its transposition into national law.

Types of survey and analytical parameters to be determined

Two types of investigation are proposed:

A) *screening activity* to evaluate the total radioactivity content of the water.

This type of investigation utilizes relatively simple protocols of analysis, and is applicable to quite a large number of samples. The analytical parameters to be determined are:

1. gross alpha activity;
2. gross beta activity;
3. tritium, if any; the measurement of this parameter is required only in cases where there are human activities sources of tritium within the catchment basin of the aquifers used for drinking water.

B) in-depth investigations to detect and quantify individual radionuclides in drinking water.

This type of investigation needs particularly complex and expensive protocols of analysis, and is applicable to a limited number of samples.

Besides gross alpha, gross beta and tritium activity – already measured during screening – the analytical parameters to be determined are:

a. uranium isotopes (mostly uranium-238 and uranium-234; the contribution of uranium-235 is normally negligible) which, together with radium isotopes, are the natural radionuclides mainly contributing to natural radioactivity in water;
b. radium isotopes (radium-226 and radium-228) because they are highly radiotoxic [2] and, together with uranium isotopes, are the natural radionuclides mainly contributing to natural radioactivity in water;
c. thorium isotopes. Thorium is seldom present in significant amounts in water and only when in the catchment basin there are rocks with high thorium-232 concentrations [1];
d. artificial radionuclides (gamma emitters, strontium-90 and plutonium isotopes), which must be investigated where local sources of anthropogenic pollution are known to be located. In this case the analysis should be carried out with sufficient analytical sensitivity. For example, when searching for gamma-emitter radionuclides by gamma spectrometry, it is normally necessary to preconcentrate sample volumes of at least some tens of litres.

In order to improve the interpretation of analytical results it may be necessary to determine the concentration of potassium-40, which is normally estimated by measuring the chemical content of potassium and using the stable isotopic abundance in the natural mixture of potassium (27.6 Bq of beta activity per gram of total potassium [2]). The complete characterization of water would also require the measurement of radon-222, lead-210 and polonium-210 concentrations, even though DL.vo 31/01 explicitly excludes these parameters.

Areas of investigation and control points

The following criteria for the selection of investigation areas at the regional level are proposed:

A) *screening investigations* should be conducted at all main regional waterworks or collection points that supply water to a significant fraction of population. However, at least three points of investigation for each province should be identified and this kind of investigations should be carried at least in each regional capital. Sampling should be preferably performed in delivery points, *i.e.* taps normally used for human consumption; a sampling is also proposed for each "supply area" (as defined in Annex II of the DL.vo 31/01: "A supply area is a geographically defined area within which water intended for human consumption comes from one or more sources, and its quality may be considered substantially uniform"). In the presence of particularly complex aqueducts, with multiple distribution centres, it may be difficult to clearly identify supply areas; in this case it is suggested to run the sampling in one delivery point for each plant.

B) *In-depth investigations* could be carried out, at the regional level, in selected areas on the basis of one or more of the following reasons:

a. in areas with known geological and hydrogeological characteristics, and where natural background levels of radioactivity are assessed to be above the regional average, for example, in terms of indoor radon concentration, radon concentration in water, natural radionuclides concentration in rocks, etc. Once the absence of known events of contamination from human activities has been verified on the basis of existing information, radioactivity should be assumed to be only natural and, therefore, in-depth investigations will be limited to natural radionuclides (isotopes of uranium and radium; thorium should be searched for only in the presence of rocks rich in thorium-232). Surveys should be planned by identifying homogeneous water supply areas of each catchment basin and distribution area, and should be preceded by a study of geological and hydrogeological features;
b. in areas where there are anthropogenic sources of artificial radioactivity that can contaminate drinking water;
c. in all cases in which the screening has shown levels of radioactivity exceeding one of the screening levels for the gross *alpha* or *beta* activity.

In-depth investigations samplings should be preferably performed in the collection points (wells or catchment points of drinking water).

Frequency of control

Screening activities. The suggestion is to repeat the sampling campaign and the measurements at least twice, in order to evaluate the range of variability of the measured values. Controls can be then repeated on the basis of a regional program.

By the way, significant changes in natural radioactivity content are sometimes observed in aqueducts fed by several supply wells when the contribution from the different wells changes. In these cases, it would be appropriate to repeat the sampling during the first year – with frequency from monthly to quarterly – in order to determine ranges of maximum fluctuation of the measured concentrations.

In-depth investigations. The frequency of controls should be evaluated according to the origin of the radioactive substances in water:

- *anthropogenic artificial radionuclide contamination* (nuclear accident fallout, environmental discharges from operating plants, etc.). It is necessary to choose sampling frequencies suitable for monitoring the temporal evolution of the phenomenon. The sampling frequency must be defined accounting for water residence times and aquifer recharge times. Frequency values should never be below those in *Table A2*;
- *anthropogenic natural radionuclide contamination* (*e.g.* presence

Table A2 | *Checking frequency in the event of water contamination from anthropogenic source. Modified from [5, 6]*

Volume of water distributed or produced each day within a supply zone		Approximate size of the catchment basin. Population served	Number of samples per year
≤ 10^5 l/day		500 people	1
> 10^5 l/day	≤ 10^6 l/day	from 500 to 5000 people	1
> 10^6 l/day	≤ 10^7 l/day	from 5000 to 50 000	1 + 1 for each 3.3 10^6 l/day and part thereof of the total volume
> 10^7 l/day	≤ 10^8 l/day	from 50 000 to 500 000	3 + 1 for each 10^7 l/day and part thereof of the total volume
> 10^8 l/day		> 500 000	10 + 1 sample for each 2.5 10^7 l/day and part thereof of the total volume

Table A3 | *Reference values for gross alpha, gross beta and tritium activity concentration*

Analytical parameter	Parameter value or Screening level	Limit of detection	Analytical method
Tritium	100 Bq/l	10 Bq/l	Liquid scintillation [7]
Gross alpha	0.1 Bq/l	0.04 Bq/l	Gross alpha counting [89] liquid scintillation [90]
Gross Beta	1 Bq/l	0.4 Bq/l	Gross beta counting [91] liquid scintillation [90]

of industrial activities utilizing materials with a high content of naturally-occurring radioactive materials, NORM). Initially, it would be good practice to repeat sampling and measurement twice at least, in order to highlight any significant changes in the radioactivity content. If the radioactivity content remains almost constant over time, controls may be repeated every two years. If the radioactivity content is time-variable, control frequency will vary from a minimum of one control per year to a maximum equal to the values in *Table A2*;

- in the absence of well-known anthropogenic contamination phenomena. When the purpose of the in-depth investigation is to characterize waters from the radiometric point of view, it may be reasonably supposed that the radionuclides in water are of natural origin; if so their variability over time will not be significant. In this case, after the initial characterization, the need to repeat controls and their frequency should be assessed at the regional level.

Reference levels

Tritium. Under the DL.vo 31/01, the tritium reference level is100 Bq/l.

Screening activities. For gross *alpha* and *beta* activity, values of 0.1 Bq/l and 1 Bq/l are proposed, respectively. If one or both these values are exceeded it will be necessary to determine any single radionuclide content contributing to the total radioactivity content. In this case, the radionuclides that must actually be searched for will be defined accounting for all available information on possible local sources of natural and/or artificial radioactivity.

Table A4 | *Reference values for the concentration of individual radionuclides*

Analytical parameter	Activity Concentration corresponding to 0.1 mSv/year	Limit of detection	Analytical method
U-238	3 Bq/l	0.02 Bq/l	Alpha spectrometry Liquid scintillation
U-234	2.8 Bq/l	0.02 Bq/l	Alpha spectrometry Liquid scintillation
U-235	2.9 Bq/l	0.02 Bq/l	Alpha spectrometry
Ra-226	0.5 Bq/l	0.04 Bq/l	Alpha spectrometry Liquid scintillation Emanometry
Ra-228	0.2 Bq/l	0.02 Bq/l	Gamma spectrometry
Th-232	0.6 Bq/l	0.06 Bq/l	Alpha spectrometry
Th-228	1.9 Bq/l	0.2 Bq/l	Alpha spectrometry
Th-234	40 Bq/l	4 Bq/l	Gamma spectrometry
Th-230	0.7 Bq/l	0.06 Bq/l	Alpha spectrometry
C-14	240 Bq/l	20 Bq/l	Liquid scintillation
Sr-90	4.9 Bq/l	0.4 Bq/l	Gross beta counting Liquid scintillation
Pu-239/Pu-240	0.6 Bq/l	0.04 Bq/l	Alpha spectrometry
Am-241	0.7 Bq/l	0.06 Bq/l	Gamma spectrometry
Co-60	40 Bq/l	0.5 Bq/l	Gamma spectrometry
Cs-134	7.2 Bq/l	0.5 Bq/l	Gamma spectrometry
Cs-137	11 Bq/l	0.5 Bq/l	Gamma spectrometry
I-131	6.2 Bq/l	0.5 Bq/l	Gamma spectrometry
Rn-222	100-1000 Bq/l	10 Bq/l	Gamma spectrometry Liquid scintillation Emanometry
Pb-210	0.2 Bq/l	0.02 Bq/l	Gamma spectrometry Gross beta counting Liquid scintillation
Po-210	0.1 Bq/l	0.01 Bq/l	Alpha spectrometry

In-depth investigations. In case investigations are carried out by measuring individual radionuclides, the concentration value – corresponding to a committed dose of 0.1 mSv/year – is calculated assuming that:

1. the water sample contains only the radioisotope under study;
2. the member of public is an adult that consumes 730 l water per year [62].

Concentration values are shown in the second column of *Table A4* for some of the main natural and artificial radionuclides.

Guidance on the requirements of Recommendation 2000/473/Euratom

In order to ensure compliance with the requirements of the European recommendation 200/473/Euratom, constant and periodic checking is proposed – at least every six months – of the water supplied by the main national waterworks, selected among those providing at least 10^8 liters of water per day (roughly equal to a catchment area of 500 000 inhabitants).

In this case the parameters to be checked are:

- tritium;
- gross alpha activity;
- gross beta activity;
- strontium-90;
- cesium-137.

Methods of measurement

Tables A3 and *A4* show some of the methods that can be used for the measurement of radioactivity in water. The list is not exhaustive: rather it aims to give some guidance to laboratories when choosing the most suitable analytical method for their purposes.

In order to orient this choice, suitable analytical methods allow to measure concentrations equal to or lower than the detection limits shown in the third column of *Tables A3* and *A4*, *i.e.*, approximately 1/10 of reference values given in the second column of the same tables.

As regards uranium-238, it is important to note that the value in *Table A3* was calculated only accounting for its radiological properties. The chemical toxicity has a reference value – proposed by WHO Guidelines [2] – of 30 µg/l (ppb), corresponding to a U-238 concentration of 186 mBq/l (much lower than the reference value concerning radio-toxicity, 3 Bq/l). Therefore, as regards uranium, the most conservative value is the chemical toxicity guidance level. The value of 30 µg/l can be compared directly with the result obtained from the chemical analysis of uranium (*e.g.*, fluorimetry or ICP-AS spectroscopy). However, if uranium is measured by radiometric methods and the activity of each radioisotope is quantified in Bq/l, it should be noted that natural uranium consists predominantly of U-238 in terms of mass; therefore compliance with 30 µg/l is verified if the concentration of uranium-238 is less than 186 mBq/l. It is also evident that, in terms of gross alpha activity, a U-238 concentration of 186 mBq/l results in at least twice as much alpha activity (at least 372 mBq/l), owing to the contribution of the alpha-emitter isotopes U-234 and U-235. Indeed, in the literature U-234 is known to be normally present in waters to an extent greater than or equal to that of U-238.

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The risk of contracting infectious diseases in public swimming pools. A review

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Summary. A review of pathogenic microorganisms presenting risk of infection in pool based artificial recreational water venues is extracted from the available scientific literature. The microorganisms are grouped both according to their way of spread and their survival and growth strategies and their characteristics relevant for the pool and spa based recreation are discussed. In order to put the proposed risks on a solid basis, among others a ten year excerpt of the waterborne disease statistics of the Centers for Disease Control and Prevention (CDC) is used throughout the article.

Key words: man-made water recreational environment, risk of infection, waterborne disease outbreaks, swimming pool, hot tub.

Riassunto *(Il rischio di contrarre malattie infettive nelle piscine pubbliche. Una rassegna)*. Viene presentata una sintesi della letteratura scientifica disponibile sui microorganismi patogeni potenzialmente infettivi, in ambienti acquatici ricreativi artificiali, come le piscine. I microorganismi sono raggruppati sia in base alle loro vie di diffusione che in base alle loro strategie di sopravvivenza e di crescita; successivamente vengono discusse le loro caratteristiche più importanti negli ambienti ricreativi considerati, piscine e terme. Per fornire adeguate e solide basi scientifiche ai tipi di rischi presentati, vengono analizzate, oltre ad altri documenti, le statistiche relative a dieci anni di osservazione di malattie legate all'acqua del Centers for Disease Control and Prevention (CDC).

Parole chiave: ambienti ricreativi acquatici artificiali, rischio di infezione, epidemie di malattie legate all'acqua, piscine, vasche da idromassaggio.

INTRODUCTION

Recreation has a substantial role in the life of ever increasing number of citizens in the world, and when choosing the scene for it, people tend to couple it with water. With evolving and advancing civilization, man-made water recreational environments are on the boom by offering health promotion and social benefits accompanied with increasing comfort and sophisticated services but also presenting to a certain extent of risk of physical, microbiological, or chemical nature. In the case of the so-called man-made water recreational environment, the extent of these risks can be reduced to a minimum by running the recreation facilities with the application of informed risk management measures [1].

According to the figures disclosed in the United States which can probably claim the greatest dimension of pool-based water recreation facilities [2], this is a solid basis for a huge market sector, and adverse health effects exerted in this environment to people or even a negative publicity may also have great economical consequences, including the direct price of health deprivation caused.

The main purpose of this paper is to review the relevant sources of infections in the man-made water recreation environment. It is neither aimed at producing a complete literature search nor dealing in depth with the methods of the risk management, but rather to summarise the most relevant pieces of information in the subject matter.

The weight of the possible adverse health outcomes of water recreation may be convincingly illustrated by specific waterborne outbreak surveillance data of the Centers for Disease Control and Prevention (CDC) – probably the largest, and continuously evolving national data collection in the subject. In spite of the considerable underestimation presumed by the compilers, 399 recreational water-borne outbreaks with more than 25 000 cases were registered in the United States in the interval from 1999 through 2008 [3-7]. Out of this number, 292 outbreaks (73.1%) with 23 800 cases (92.6% of all) were attributed to "treated recreational venues", *i.e.* pool, spa and similar facilities. Below, when looking into the aetiology of recreational water-borne infections, several reference will be made to this unique data pool [8] collected for a good deal of the population of the most developed part of the world.

Numerous infectious agents – bacteria, fungi, viruses and protozoa – may threaten the health or comfort of pool and spa users. Characterization of

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these pathogens according to the source and mode of transmission may be the key to the efficient way of risk management.

PATHOGENIC AGENTS OF FAECAL-ORAL SPREAD

Most classical and emerging waterborne pathogens are of faecal-oral in their mode of spread and thus share ill reputation in bathing and drinking water. In principle any microorganism capable of spreading by the faecal-oral mechanism can be involved in pool waterborne infection as water is an excellent vehicle for them. According to the source of infection, they can be both zoonotic and solely human pathogens with the direct source generally being another bather who shared the pool with the affected person, though much more rarely incidental cases and clusters can derive from infected animals which could access the pool or its close surrounding.

Most of the symptoms caused by them are enteric in nature but a couple of the microorganisms spreading this way can give rise to other types of diseases with serious generalised or localised neurologic, cardiologic respiratory or other manifestations or in other cases the primary, acute enteric disease may evolve into a secondary phase with a multitude of manifestations in other organs of the body [9].

The risk of contracting infection depends on the actual probability of the agent's incidence in the population usually visiting the pool and spa facilities. Pathogens which has never been detected or can be reasonably held exotic are out of the considerations targeting risk treatment measures while those that are frequently encountered in the population should be in the forefront of these measures.

Among the aspects rendering one or other pathogenic microorganism of possible incidence more or less likely to be actually getting involved in an outbreak are a couple of their inherent characteristics, like virulence and infectivity, rate and duration of post infectious shedding, environmental robustness and susceptibility to water disinfection.

Risk avoidance measures therefore should always be well informed about the incidence of relevant pathogens and their nature as to their aforementioned characteristics. If the risk connected to the most robust ones is appropriately addressed, that of others of similar nature but less vigour will more or less automatically be contained. Unlike in the case of non-treated natural bathing waters, the risk of infection by the management is addressed by actions targeting the removal of pathogens supposed to already be present in the water rather than preventing them in getting there. Nevertheless growing importance is given to measures which try to positively influence the users' hygiene behaviour in order to avoid introduction of the disease agents.

The source of these pathogens is almost invariably the ill or asymptomatic carrier who will shed them from the contaminated orifices and adjacent skin surfaces, although less frequently accidental faecal release (AFR) and vomit may be implicated [1]. Occasional cases may be caused by pools contaminated by infected animals having access to the pool.

The faecal-oral pathogens represent numerous bacteria, viruses and protozoa of widely different characteristic, with their infectivity (discussed mostly as attack rate) and their availability to water disinfection being consideration of utmost importance. Enteropathogenic bacteria tend to be more susceptible to disinfection and most of the outbreaks caused by them through pool water can more or less certainly attributed to the lack or gross inadequacy of the water treatment. On the other hand, in environments equipped with improved technology of pool sanitation, presently far most prevalent is the most robust pathogenic protozoon with high resistance to chlorine [10]. Given its outstanding epidemiological significance, *Cryptosporidium* will be discussed in a separate article.

Most publications on recreational water-borne outbreaks report cases from developed countries. It may be a true over-representation but there may be arguments for the outbreaks of this like being much more infrequent in the developing world. Enteric bacterial and viral infections' spread via public pool facilities could in principle present high risk in developing countries since the regulations and the accessible technical level may not raise real barrier to them. However, access to these facilities is more limited in these countries and thus the population low chance of to either shedding or contracting the pathogens via pools. For the developing countries, the major challenges in combating acute gastrointestinal infectious diseases are still the lack of safe drinking water and the low general hygiene standards.

Another, although scarcely documented factor may be the diverse likelihood of acquiring immunity to the infection by an enteric pathogen. An infection may confer protective immunity of very different duration (cfr. infection by hepatitis A virus and that by norovirus). Immunity is frequently limited in being protective by the genetic variability of the pathogenic agents – a characteristic *e.g.* of noroviruses [11]. Some of the emerging pathogens, most notably *Cryptosporidium*, have been shown to trigger an immune response [12] boosted during repeated infections thus rendering the risk of cryptosporidiosis less imminent. This effect may further be amplified by objective and subjective factors limiting persons suffering from self-limited gastro-intestinal infection in seeking medical assistance and being accessible for epidemiological investigation. In regions and countries where enteric pathogens of more severe health impact are highly endemic and even frequently epidemic, the effect of those having the greatest bearing in the developed world – like *Cryptosporidium* and norovirus may be much lesser. These factors may present to a certain extent explanation to the known big difference of

the cryptosporidiosis incidence even in the northern hemisphere.

To illustrate the distribution of prevalence of pathogenic agents in recreational waterborne outbreaks in a highly developed country, below frequent reference is made to the afore mentioned ten-year segment of the statistics compiled by the Waterborne Disease and Outbreak Surveillance System (WBDOSS) maintained by the CDC of the USA. During 1999-2008 an overall number of 166 outbreaks of acute gastro-intestinal infection (AGI) associated with treated recreational water venues (*i.e.* swimming and other pools and similar environments) were reported. This made out 72.5 percent of the total of 229 AGI outbreaks reported for all recreational waters, and 41.4% of the total of 399 recreational water-borne outbreaks, indicating the weight of risk for the pool and spa user community. While bacterial and viral pathogens were only responsible for 23 outbreaks (13.9%) and 663 (3.1%) cases respectively, the highly chlorine resistant *Cryptosporidium* dominated the statistics with 123 outbreaks (74.1%) and 18839 cases (90.2%). Another valuable source of information is an account of 12 years waterborne outbreaks in England and Wales between 1992 and 2003, reviewing 89 reported outbreaks of waterborne infectious intestinal disease affecting 4321 persons [13]. Swimming pools and similar facilities were implicated in 35 (39%) of these outbreaks affecting 762 persons. Unlike in the USA only parasitic protozoans (32 times *Cryptosporidium* and twice *Giardia* and *Cryptosporidium*) were implicated in the referred British swimming pool outbreaks.

Bacteria

Faecally derived bacterial pathogens, once dominating the waterborne outbreak reports seem to be represent an altogether low risk. Some of the ill-famed ones, like Salmonella species, including *S. typhi*, *S. paratyphi* seem to not at all being implicated in recreational water-borne outbreaks, though continue to be significant risk for drinking water outbreaks in the developing world [14, 15]. Rather than the survival capability in water, it is the infectious dose that may predispose an agent to be capable of effective spread and lending an attack rate necessary to trigger an outbreak in recreational waters. Recreational waterborne enteric pathogens stand out by extremely low infectious dose – typically in the range of 10 to 200 infective units.

The single most significant factor preventing enteric pathogen bacteria and viruses is the effective disinfection of the circulating pool water, as disinfection practices have been formulated just on the basis of experiences with them and reinforced by monitoring for indicator bacteria of similar sensitivity to disinfectants. Small wonder that all outbreaks linked to treated recreational waters (except for cryptosporidiosis) have been shown to be associated with gross treatment inadequacies and especially missing or ineffective disinfection. Venues which are not regulated, or regulation is not usually enforced are presenting typically a higher risk of infection. New types of man-made recreational water facilities, like interactive fountains or bio-ponds without disinfection have been shown to be emerging source of infection [7]. The pathogens still on the stage are those that stand out according to infectivity and still can be found circulating in the population even if at low prevalence.

The most important bacterial species of all seems to be *Escherichia coli*, which – though being the epitome of the commensal enteric bacterium – equipped with either of a couple of virulence factors is able to produce diseases of diverse severity from a short self limiting diarrhoea to frequently fatal haemolytic-uremic syndrome. It is the Shiga-Toxin producing (STEC) or verocytotoxin producing strains (VTEC) or by the widest known designation the enterohaemorrhagic *E. coli* (EHEC), that are almost exclusively implicated in waterborne outbreaks. Persons who have only diarrhoea usually recover completely but children under 5 years and the elderly are more frequently endangered by a complication called haemolytic uremic syndrome (HUS) – a life-threatening condition characterized by haemolytic anaemia and renal failure – that may occur in about 2-8% of infections. Secondary spread in man is common [16]. *E. coli* O157:[H7] is the most widely recognized VTEC serotype, but non-O157 EHEC strains are more common in most continental European countries and Australia. In contrast to the majority of diarrhogenic E coli strains known to be of solely human origin, *E. coli* O157 have been identified as zoonotic strain of bovine origin with several mechanisms of secondary spread, including waterborne events. The largest European outbreak in spring, 2011 was caused by *E. coli* O104 with predominantly foodborne origin, and mostly hit Germany with more than 4300 cases [17]. It is an indirect acclamation of the German level of pool hygiene and risk management that no secondary, pool-waterborne cases have been reported during the rather extended outbreak. EHEC is communicable for duration of fecal excretion (7-9 days) extending to up to 3 weeks in one third of children [18].

Recreational water related cases have sporadically been reported in the North America and in the United Kingdom and pool-borne ones are always linked to small paddling pools and other, small non-chlorinated facilities [19, 20]. A review of 20 years *E. coli* O157:H7 epidemiology in the USA revealed a total of 350 outbreaks with 31 (9%) recreational water related ones, a third of which linked to swimming pools [21].

Shigella has only humans and primates as natural host and causes in them symptoms from mild abdominal discomfort to dysentery, a serious condition characterized by cramps, tenesmus, diarrhea, fever. Some strains produce enterotoxin and shiga toxin that may cause haemolytic uremic syndrome similarly to some *E. coli* strains. Mucosal ulceration, dehydration and rectal bleeding can lead in neglected cases to death, as it happens according to the estimations of WHO in 108 000 cases a year

[22] mostly among children in the developing world. *Shigella flexneri* is another bacterium renowned as causing sequel like Reiter's syndrome in genetically disposed people [23]. The most frequent route of infection is via food or water contaminated with the faeces of shedding persons, by hand-to-mouth infection or via fomites and mechanical vectors, like houseflies. The incubation period is half to four days and the median duration of the disease is 5-7 days [24]. Though not more than 100 bacteria is enough for infection [25], water-related dysentery outbreaks more recently are rather rare in the developed countries. Infrequent outbreaks are reported from the USA, but most of them are caused by contaminated natural bathing waters sometimes in combination with other enteric pathogens [26]. Shigelloses linked to treated recreational venues have been reported in 7 occasions to the WBDOSS in the period of time chosen for analysis. All but one of them were caused by *S. sonnei* and involved 178 persons contracting the disease either in small, drain-and-fill type wading pools or residential pools, or when having fun with interactive fountains with missing or inadequate chlorination.

Campylobacter is a widespread zoonotic pathogen with the most prevalent species. *C. jejuni* is the estimated leading cause of acute infectious diarrhoea in most industrialised countries. It is overwhelmingly food-borne but certain outbreaks have been attributed to contamination of drinking water. *C. jejuni* infection is characterised by diarrhoea and is generally self-limited resolving in a couple of days (3-7 days). Serious complications of the infection may occur in about 1 out of 1000 infections with the most frequently occurring Guillain-Barré syndrome (see above) which have an onset several weeks after the diarrhoeal illness and lasts for several weeks to months.

Although infectivity of this bacterium seems to be somewhat lower than EHEC or Shigella, requiring about 10 000 cells to swallow [27], waterborne outbreaks occur relatively frequently [13, 28]. Poolwaterborne outbreaks on the other hand are rather rare, a reason for which may be that *C. jejuni* is more sensitive to chlorine than most other waterborne pathogens [29], rendering them more easily controllable by normal disinfection practices. The only reported pool waterborne cases can be traced to minor semi-public or private facilities, where even the minimum requirements of pool safety were unobserved. In a case of pool water mediated outbreak caused by *C. jejuni,* frequent presence of ducks was indicated by the investigators [3].

Viruses

In contrast to decades back in time, presently the viral outbreaks seem to outrange those of bacterial origin. It may be due to the modern molecular diagnostic techniques, but at least partially a true increase in incidence must have occurred because of changes in the epidemiological situation and the technology involved in the water recreational environment, favouring to the spread of more robust and contagious viruses. Adenoviruses, noroviruses, human enteroviruses hepatitis A and E virus and astroviruses are found most frequently in the literature as plausible viral causative agents in recreational water setting. For all of viral causative agents of waterborne outbreaks the major route of spread is the faecal-oral, though some adenovirus groups may infect by other mechanisms.

The etiological pattern of waterborne viral outbreaks is strikingly different from the general community outbreaks. Out of the three major virus groups – rotavirus, norovirus and adenovirus – causing the overwhelming majority of acute gastroenteritis diseases worldwide, only norovirus seems to have really significant role in the causation of recreational waterborne gastrointestinal diseases.

Adenovirus – a double-stranded DNA virus – has significant share in the burden of infections in the recreational water setting. Adenovirus comprises 54 human related serotypes [30], a third of which are incriminated in human diseases with gastrointestinal, respiratory, ocular, urinary and neurological manifestations [31]. Many adenovirus serotypes multiply in the small intestines and are shed in the faeces, but only 40 and 41 are unequivocally associated with gastroenteritis. Most adenovirus infections are mild or asymptomatic, except for those acquired in early childhood when it is second to rotavirus as a cause of childhood gastroenteritis. Human adenoviruses could be appropriate indicators of the presence of human viral pathogens in the environment [32] being more prevalent than enteroviruses due to their high stability in the environment [33]. Adenovirus 40 and 41 – found to be in the highest number of all studied adenovirus and enterovirus types in polluted water [32] – were postulated to have impact by drinking and recreational water outbreaks, but neither of them have been found in any of them to date, although frequent exposure is beyond doubt [34]. The only adenovirus outbreaks found in the literature are incidents of pharyngoconjunctival fever, caused by either of serotypes 3, 4, 7 or 7A, the most prevalent adenoviral agents of upper respiratory tract infections. Notably almost all of these waterborne outbreaks have been linked to pool setting calling the attention to specific routes of infection by them. Beyond the typical route of ingestion water contaminated with faeces or other excreta containing viable virus particle, simple rinsing of the throat or conjunctiva, exposure to airborne droplets of contaminated spray, or even direct airborne person to person infection in the crowded setting can be endorsed. Of the reviewed 55 recreational waterborne viral outbreaks selected by scientifically sound inclusion criteria from the literature of 55 years between 1951 through 2006 [35], 13 were caused by adenovirus and 11 of the latter was linked to pools, once qualifying adenoviruses the number one viral agent in pools. The reviewers hypothesis of the decline of incidence of pharyngoconjunctival fever outbreaks

over the decades examined seems to be supported by the data retrieved by the WBDOSS in the USA from 1999 to 2008, when none of the outbreaks linked to treated recreational water venues were caused by adenoviruses. Since then another case with 59 affected children caused by human adenovirus serogroup 4 was published in Spain [36].

Two further virus groups can be highlighted as those causing the highest number of enteric infections worldwide. Rotaviruses – a double stranded RNA virus group – are the leading causative agent of severe diarrhoea among children with a very high death toll in the poorest part of the world, having killed 527 000 children under 5 year age in 2004 [37]. The incidence of rotavirus infection is similar in developing and developed countries and in the prevaccination era 95% of the children were estimated to experience rotavirus infection by the age of 5. Reinfection can occur at any age and subsequent infections are generally less severe due to progressive immune protection [38], with mostly subclinical infection in adults.

In contrast to rotavirus, norovirus (single stranded, small-round structured RNA viruses; on earlier names: Norwalk virus or calicivirus) may cause gastroenteritis in any age and in fact are the most prevalent cause of it in the world with an estimated one billion case of acute diarrhoea a year. In the USA an estimated number of 21 million illness, 70 000 hospitalization and 800 death, is the toll of norovirus [39]. It mainly causes 1-3 day long self-limiting diarrhoea after 24-48 hours of infection, but severe symptoms may be manifected in early childhood and in elderly persons. Around 30% of infections from norovirus are asymptomatic, but the infected persons shed the virus. It is highly contagious, and less than twenty virus particles can cause an infection [40]. Although both rotavirus and norovirus can be implicated in water contamination incidents leading to waterborne outbreaks, there is great difference between their real involvement. While norovirus is found to play significant role in waterborne outbreaks [41], evidence for rotavirus causing such outbreaks is rather sparse [42, 43], though it was occasionally found together with other pathogens in case of gross contamination [44]. The difference is even more marked for recreational waters and especially for pools and similar environments. Indeed, rotavirus is missing from the review of Sinclair, *et al* [35]. Norovirus outbreaks are however abundantly demonstrated in the literature since the early detection of this virus. Out of 55 definitely recreational waterborne outbreaks studied between 1951 and 2006, 25 (45.5%) was found to be caused by norovirus giving the highest contribution out of the six virus groups considered. Out of these outbreaks 7 (28%) was linked to pool water. Rotavirus was not found by WBDOSS among causative agents in poolwater in the USA between 1999 and 2008, while norovirus caused 9 outbreaks (n.b. there is a considerable overlap in both cited pool of outbreaks). An explanation for this can be the distinct pattern of immunity between both types of viruses. As above pointed out, protective immunity against rotavirus infection is universal after the first couple of life years in contrast to norovirus which does not induce lasting immunity [45] among others because of the great genetic variability of the genome of the virus.

Astrovirus, another small single stranded RNA virus is ranking the fourth most common known cause of viral gastroenteritis. Infection is rather common already in the early ages of life, and generally causes very mild self limiting diarrhoea or even more frequently remains asymptomatic. Although significant role is attributed to contaminated water in its spread, only one waterborne astroviral outbreaks have so far been revealed [13]. In the peer reviewed literature the single mentioning of astrovirus in association with recreational water was published in Finland, where it was found both in the water of an outdoor wading pool and the patients' stool specimens together with norovirus [46].

Another couple of viruses of predominantly faecal-oral route of infection are occasionally also implicated in recreational pool waterborne outbreaks but they are distinct by causing diverse clinical manifestations other than symptoms of acute gastroenteritis. A group of small single stranded RNA viruses of the Picornaviridae family are the enteroviruses classified into four groups for A through D comprising poliovirus, coxsackievirus A and B (both with several serotypes), echovirus (with 6 serotypes) and enterovirus 71. From the present review, Poliovirus, once having caused the most feared outbreaks of poliomyelitis can be excluded on the basis of its almost complete eradication. Though most of these viruses are entering the body via ingestion, multiply in the intestinal tract and are shed with faeces, gastrointestinal illness is the least characteristic disease they can cause. The wide array of symptoms they cause, extend from neurological, through cardial, conjunctival, respiratory and dermatological manifestations, although most infections result in mild or asymptomatic illness, with the highest incidence in children. The incubation period is usually less than 5 days and the infected persons frequently shed the virus before the symptoms emerge and continue to shed until several weeks after recovery. There are global or at least continental epidemiological trends with the emergence of one or more serotypes of enterovirus and given their great contagiosity, the high cross-infection rate of the actual variants quickly establishes dominating prevalence of them [47]. Out of the 55 recreational waterborne outbreaks referred by Sinclair *et al.* [35] 12 was caused by enteroviruses (coxsackie and echoviruses) and 7 of them (all caused by echovirus types) could be linked to swimming pools. The ten year surveillance data of the CDC WBDOSS refer only one enterovirus outbreak (echovirus 9), which is also covered by the above referred review.

Two further viruses both causing hepatitis in infected persons are finally considered as pathogens possibly causing recreational water outbreaks. Hepatitis A virus (*Picornaviridae* family) and hepatitis E virus

(*Hepeviridae* family) are unrelated though the infection by both of them induces similar, generally mild symptoms of hepatitis, or even more frequently, particularly at childhood infections, remains symptomless. Hepatitis viruses are very contagious and their spread has a distinct pattern depending on the general hygiene circumstances. Waterborne spread of both viruses are well documented, with major drinking waterborne outbreaks of hepatitis E in Asia [48-50]. Although several authors isolated Hepatitis A virus from surface waters, which were used for recreation (*e.g.* [51]), outbreaks related to recreational water are relatively rarely reported. Long incubation period may contribute to the difficulties the detection of these outbreaks. Both the reviews of Sinclair *et al.* [35] and Pond [52] cites three credible pool-related outbreaks worldwide, and none have been identified by the CDC WBOSS team between 1999 and 2008.

Protozoa

In addition to *Cryptosporidium*, which is the most important cause of waterborne outbreaks in the developed part of the world, the only significant protozoan agent to cause recreational waterborne outbreaks is *Giardia duodenalis*, a unicellular parasite that infects humans and a range of wild and domestic animals. Giardia is capable of surviving long environmental exposure in the form of resistant cyst and giardiasis is the most frequent intestinal disease worldwide caused by a protozoan estimated to cause about 300 million cases a year [53]. Waterborne spread is one of the most significant mechanisms of Giardia infections [54]. Very few cysts can establish infection, though the majority of infected persons remain asymptomatic cyst shedder and most of the rest will experience a self limited acute diarrhoea lasting 1-3 weeks [28]. Symptomatic disease ranges from mild diarrhoea to a severe malabsorption syndrome [55]. Asymptomatic cyst passage can last as long as six months [56]. Giardia is relatively resistant to disinfection processes, and its risk is confirmed by several poolborne outbreaks and surveillance data rewieved by Pond [52]. In the years 1999-2008 six giardiasis outbreaks with 216 infected persons linked to pools were reported to the CDC WBDOSS. In half of these outbreaks mixed pathogenesis with cryptosporidia were revealed.

Microsporidia, a large phylum of highly specialised obligate intracellular parasites with more than 700 species are infecting a range of divergent hosts from insects to mammals including humans. Once thought to be protozoans, more and more authors bring reasons for their identity as fungi [57]. They are considered as the fourth most prevalent protozoans causing diarrhoeal disease worldwide (with an estimated 30 to 70% infection ranges [58]), though primarily immunocompromised persons are at highest risk of severe disease. The infection with Microsporidia can result not only in enteric disease but a number of other organs, from the cornea to the kidney and central nervous system can be attacked. The most frequently found species involved in human pathogenic process are *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*. Although only few data are available to confirm their connection with pool waterborne infections, both their worldwide distribution in water also for different human uses as reviewed by Pond [52] and occurrence as highly resistant spores capable to survive even several years in wet environment, predispose them to be considered as emerging pathogens of this sort.

PATHOGENIC AGENTS WITH OTHER THAN FAECAL-ORAL SPREAD

There are several groups of microbes that can spread by other than faecal-oral route and cause diverse diseases or conditions other than gastrointestinal. Some bacteria are capable to exist both in saprophytic and parasitic life-cycles, and man-made recreational water environment offers appropriate niches where they thrive in microbial biofilm communities until opportunity opens for entering a parasitic cycle in humans.

For a couple of other organisms recreational water venues provide merely sites of passive transfer from carriers to new hosts when opportunity opens to find the way of entering it. They can incidentally infect susceptible hosts mostly by way of their contacting contaminated surfaces. Mediating environment can be physical objects in and around the pool and its facilities (steps, rails, pool-bottom and walls), fomites like slides, benches, sunbeds and surfaces of the ancillary facilities (toilets, locker rooms, etc.).

Biofilm mediated organisms

One of the most frequently reported pathogen in the man-made recreational setting and the one requiring doubtless the highest death toll is Legionella.

The Legionella genus consisting 57 species [59] of an ubiquitous aquatic organism [60] has recently been identified as causative agent of actual and earlier outbreaks of respiratory diseases, commonly called legionelloses. The gravest syndrome, legionnaires' disease – a severe pneumonia – can attack anyone but has the highest death toll in elderly or immunocompromised persons and those suffering underlying conditions like diabetes, alcoholism and other chronic diseases where mortality can be as high as 30-40%. The type species called *L. pneumophila*, and first of all its serotype 1 (out of 16 identified to date) is responsible for far the most pneumonia cases with further 23 species associated with human disease [61]. Another form of legionellosis, a non-specific upper respiratory set of symptoms (Pontiac fever) is self limiting and short duration febrile flue-like illness that has been associated with exposure to *L. pneumophila*. In contrast to legionnaires' disease, Pontiac fever has a very high attack rate affecting up to 95% of the exposed persons [62]. Incubation period is 2-10 days in case of the legionnaires' disease and a few hours to 2 days in Pontiac fever [63].

Legionella raised scientific interest not only because of being a dangerous emerging pathogen, but also by its particular "dual life cycle", offering experimental model for molecular biological research into the bacterial intracellular differentiation. Legionella is ubiquitous in natural water bodies and wet soil and its success in survival and competition is ensured by its ability to infect protozoa and proliferate in them – the parasitic mechanism also utilized in the pathomechanism of legionnaries' disease as human pathogen when it parasitizes the alveolar macrophages. Its persistence in man made water environments is linked to biofilm communities [64] where it can establish itself and at a temperature range of about 25-45 °C has a solid selective advantage in the competition [65]. Symbiotic or parasitic growth in free-living protozoans have been shown to yield further benefits to Legionella, not only by conferring protection against harsh environments like biocids, but also imparting higher invasiveness and human pathogenicity [66]. There is though some controversion in the scientific literature whether Legionella can multiply in biofilm without having recourse to parasitic multiplication in amoebae [67], but there is no doubt about the significant selective benefits of this mechanism for the organism [68].

Transmission of Legionella takes place via aerosols containing droplets of diameter of less than 5 μm. The infective dose is small and the circumstances of exposure have a definitive role ensuring it arrives to the site of entry. There are a couple of anthropogenic water-based environments that frequently serve as a niche of Legionella multiplication and a basis for subsequent human exposure. Next to evaporative cooling systems and extended hot water networks in large buildings, pool and spa setting is the third most significant setup where the most infection occurs. All three share characteristics that support Legionella and the associated biofilm organisms in proliferating and reaching human hosts: large, relatively warm, stagnant or slowly circulating water bodies in contact with extended surface and carrying dissolved and suspended nutrients. The biofilm once established and matured, offers excellent protection against adverse environmental effects, and trough sloughing gives rise to suspended cells that find way through specific sites of exposure to the host. This provides the key to measures of risk avoidance in the case of whirlpools, spa pools or hot tubs and similar facilities that are far the most frequent risk sources in recreational setting, where the establishment of the biofilm is the key factor rather than the physical circumstances of the exposure. Exposure and establishment of infection does not necessarily mean disease: most people will not develop clinical symptoms even in the evidence of exposure [69]. Due to the intrinsic characteristics of the spread of the legionnaires' disease, it mostly occurs as sporadic cases and small clusters, although large outbreaks also occur – mostly in conjunction with contaminated cooling towers [70].

There are abundant sources in the scientific literature proving the great burden of legionellosis diseases [1, 27, 61, 69, 70]. In spite of the great efforts of surveillance, the disease remains largely underreported due to the difficulties of revealing sporadic cases, and because its symptoms are rather unspecific and respond well in most cases without exact diagnose to antibiotic treatment. The incidence of reported cases of legionellosis in the USA in 2009 was 10.8 [71] and in Europe (for 25 EU member states, Norway and Iceland) 11.2 per million of population [72]. The true incidence however is estimated 100 per million. Out of this relatively large number of cases in Europe, only 254 pertained to 101 clusters. As environmental investigation is generally lacking, the mode of infection beyond its being travel associated cannot be resolved, and there are no data in the annual reports on the proportion of cases and clusters caused by pool and spa facilities. They present however principal risk during travel and this may have extended with the growing popularity of such facilities in hotels and other sites of accommodation. The most recent cluster of travel associated legionellosis in Calpe, Spain with 40 cases during more than half a year turned out to have been caused by a spa pool [73].

In the United States, 24 pool-linked outbreaks were reported to the CDC WBDOSS, in the reviewed time span, 5 of which was outbreak of Pontiac fever alone or mixed with cases of legionnaires' disease. Pond reviewed altogether 30 outbreaks of legionellosis linked to recreational waters and found that the greatest risk of infection has been linked to either thermal spas where no disinfection is allowed or hot tubs/spa pools [52]. Display hot tubs operated without disinfection have caused severe outbreaks on several occasion, evidencing that the risk of infection is high without immersing in the contaminated water [74-76]. Although Legionella bacteria were incidentally isolated from swimming pools, they have never been identified to cause legionellosis, except for cases linked to poolside showers [77]. As a specific subset of travel associated cases, spa pools on ships are also frequently implicated in causing legionnaires' disease [78]. When looking at the circumstances that lead to infections of Legionella in pool and spa facilities, mismanagement and first of all flawed disinfection seems to be the major triggering factor [63] calling for more efforts of control and supervision.

Pseudomonas aeruginosa – the most frequently referred to opportunistic pathogen bacterium in connection with the pool and spa environment – is a nutritionally highly versatile, ubiquitous aquatic bacterium capable of adapting to various environmental conditions including water, vegetation, soil and various niches of the human body [1, 79]. Although not belonging to the typical resident skin microflora and infrequently colonizing (2.6 to 24% depending on the location tested) in non-hospitalized adults [80], *P. aeruginosa*, may yet be considered a normal constituent of the human natural microflora [81]. Besides silent colonisation, as a tough opportunistic pathogen armed with a number of virulence factors and

antibiotic resistance [82-84], it can overtly infect humans mostly with compromised immune system but not infrequently also healthy ones [85, 86]. The most prevalent location of infection originated in pool and spa environment in healthy people is the skin and especially that of the outer ear canal, with related clinical symptoms like swimmer's ear (otitis externa) and hot tub rash (folliculitis). Reports in the peer reviewed and grey literature give extensive description on outbreaks of otitis externa, folliculitis and/or dermatitis rash with variable severity and duration of up to > 6 weeks [87, 88]. In the interval between 1999 and 2008 a total of 52 outbreaks of dermatological infection by *P. aeruginosa* were reported with 955 cases. Thus *P. aeruginosa* is ranking as the second most prevalent pathogen after *Cryptosporidium* in the pool and spa environment in the USA. The true incidence of whirlpool-associated P. folliculitis is difficult to determine because the symptoms are often mild and self limited, and the patients frequently do not seek medical attention [89].

Otitis externa is characterized by inflammation of the external auditory canal. Factors increasing the risk of otitis externa related to water exposure include amount of time spent in the water prior to the infection, a record of previous ear infections and repeated exposure to water by slackening the protective wax coating of the outer ear canal [90, 91]. Another specific manifestation called hot foot syndrome – a clinically distinct painful erythematous plantar skin eruption – was found to be due to high densities of *P. aeruginosa* in a pool with an abrasive flooring [92]. Occasionally, *Pseudomonas* infection may give rise to more serious consequences like corneal ulcer or wound infection, respiratory system diseases and urinary tract infections [93]. Faecal and non-faecal shedding from humans is suggested to be the major source of *P. aeruginosa*, but in fact the importance of the primary source is often outdone by the conditions favourable to its growth in attached biofilms in and around the pools. Such locations may be various pool structures (linings, decks, drains, filters) and surrounding objects and fomites from the benches to towels and children's toys [94]. The connection of the abundance of *P. aeruginosa* sources and occurrences with the intermittent incidence of documented health damages and outbreaks is still not well understood. Rather then a classic dose-response relationship which would indicate a highly variable risk to healthy individuals, a variety of related factors like the contact time, the biotype and virulence of the implicated strain and a couple of personal conditions of the bathers (like time spent and repeated exposures in water, the water temperature, history of earlier infections, etc.) has heavy impact on the probability of getting ill from a *P. aeruginosa* infection [94]. Several authors assess the circumstances that lead to outbreaks and they agree in finding inadequate or completely missing disinfection the main factor [7, 91]. The effect of chlorine repeatedly dosed in the form of cyanurates may largely decrease due to cyanurate-lock – a contraindication of using these compounds in whirlpool spas. Sometimes outbreaks of *P. aeruginosa* (and also Legionella) infection are epidemiologically linked to both the swimming pool and the spa pool of the facility [7]. Experiences of the CDC WBOSS team prove contributing conditons like exposure occurring in a hotel/motel setting, in which spa operation is not a full-time job and exposure in the context of a group event when crowding may quickly deplete disinfectant levels.

Several non-tuberculotic *Mycobacterium* species (like the *M. avium-intracellulare Complex /MAC/, M. chelonei, M. fortuitum, M. gordonae, M. kansasii, M. marinum*) were detected in various water environments and described to be involved in various pathologic processes [95]. The environmental mycobacteria are biofilm-associated [96] and are proposed to profit from endosymbiotic relationship with protozoa where they may enter into more virulent status [97]. In the majority of cases waterborne – and among them pool-related – mycobacterial infections present a risk for persons of compromised immunity or other underlying condition, like open wounds, cystic fibrose, etc. [98, 99]. Atypical mycobacteria have also been frequently isolated from healthy individuals [100] and are frequently called "leisure-time pathogen", referring to the fact that the infection is usually associated to activity during water recreation. The most frequent form of the infection in healthy individuals is a self-limiting form of skin granuloma on prevalent locations like elbow, knee or wrist and may also occur in epidemic form involving several users of swimming pools. The infection is frequently linked with minor skin abrasions. First of all *M. marinum* is associated with swimming pool granuloma outbreaks [101]. Respiratory system disorders (hypersensitivity pneumonitis, hot tub lung) have also been associated with mycobacteria, first of all with MAC mostly in connection to the use of poorly maintained and non-disinfected spa pools [102, 103].

Several types of unicellular protozoa (free living amoebae, FLA) have been described that thrive in abundance in natural or artificial water environments but are also able to invade at variable ports human hosts and cause severe syndromes. They are all characterised by favouring higher ambient temperatures close to that of the human body and by changing lifecycles adjusted to the circumstances. The FLA of the worst fame is *Naegleria fowleri* which can inflict primary amoebic meningoencephalitis (PAM), an almost always fatal condition. *N. fowleri* is often found in warm waters used for recreation and curative purposes, but less frequently in treated recreational waters. Naegleria can exist in three different appearances: its dangerous amoeboid trophozoite form is its reproductive (and frequently invasive) format. Transitionally it lives as a motile flagellate or in unfavourable conditions it stays inactive as an environmentally resistant cyst capable

to hatch (excyst) whenever circumstances turn favourable for feeding and reproduction. The trophozoite form enters the body of the host via the nasal mucosa – frequently under hydraulic forces when jumping into the water or diving – and invades the brain via the olfactory nerve. The disease is generally fatal about 3 to 10 days after the onset of the symptoms which in turn follows the infection in 7-10 days [104]. The number of cases seems to grow year by year, and though most of them are linked to natural waters (*e.g.* small warm ponds) pool-associated cases are also published. A cluster of cases happened about 50 years ago in a single indoor pool in Northern Bohemia where 16 young patients died in fulminant PAM caused most probably by *N. fowleri* [105]. Since than several hundred cases were identified which were mostly associated to natural waters but incidentally thermal pools have also been involved [104].

Another protozoan frequently described as water associated pathogenic agent is Acanthamoeba. This FLA is rather often found in recreational waters and among them in swimming pools [106], and is responsible for about 5% of the contact lens related microbial keratitis cases. Though most cases are linked to using unsterilized contact lens fluids, swimming in contact lens is described as a risk factor to be avoided [107]. Another serious disease caused by some genotypes of Acanthamoeba is the granulomatous amoebic encephalitis (GAE) a subacute or chronic but invariably fatal brain infection. The route of infection is thought to be by inhalation of amoebae or introduction through skin lesions. Unlike with amoebic keratitis, victims of GAE are mostly persons with conditions compromising the immune system, though GAE cases of immunocompetent children and adults were also described. In contrast to N. fowleri, no clearly swimming pool related GAE cases have been reported to date though its potential risk is apparent in the presence of FLA of proven pathogenicity [108].

Biofilm related conditions not coupled with a specific microorganism

Respiratory symptoms impossible to clearly link to a specific microbe are incidentally associated with pool and spa setting and are attributed to allergenic reaction to inhaled bacterial endotoxins. Outbreaks of granulomatous pneumonitis among lifeguards have been associated to occupational exposure to aerosol generated by indoor pool-related water features with high numbers of bacteria (*Pseudomonas aeruginosa* and others) in the water and high concentrations of endotoxin [79, 109]. Beyond published cases, anecdotal incidents also crop up from owners of residential whirlpools. The cases are always associated to gross neglect in maintenance and missing disinfection with consequential very high bacterial counts in the water of the whirlpool. These phenomena are also clearly associated to pool-related biofilms and their prevention needs measures just like risk management for infections caused by specific biofilm microorganisms.

Infections by microorganisms of other than faecal-oral spread by way of passive transmission

For a complete overview of microbiological risks in the man-made recreational water installations, consideration should be given to a diverse group of non-faecally derived microorganisms that are carried by persons – or incidentally by animals – with or without symptoms of various infections and shed into the water or onto surfaces of objects in the pool and spa facilities and may infect susceptible hosts by plain encounter. They present temporary nuisance rather than serious disease, and are generally sparsely referred by the scientific literature but still may present a considerable social and health burden. In contrast to the majority of these, Leptospira, the only relevant zoonotic agent that may be accidentally introduced by an infected animal into a man made recreational water can cause serious, and even fatal disease. The Leptospira genus consists of several saprophytic, intermediate and pathogenic species, and out of them *L. interrogans* – and first of all its serovar *L. icterohaemorrhagiae* – causes the most severe form of leptospirosis (Weil's disease). Leptospirosis has rather variable clinical manifestations from a mild flu-like course to the severe, often fatal icteric form characterised by kidney and liver failure. The source of the infection is always an animal host, in case of *L. icterrohaemorrhagiae* infections most probably rats, that excretes the bacteria with it urine. Leptospira is rather sensitive to environmental stress but on the other hand extremely infective: a very small number of bacteria (1 to 10 cells) can establish the infection by various ways of entry. It can penetrate the skin on small abrasions and the mucous membranes if swallowed, inhaled, or contacted (*e.g.* conjunctiva). There are several reports of waterborne leptospirosis outbreaks but almost invariably in conjunction with bathing in natural freshwaters. Pool water related cases are probably very rare; two outbreaks reported to date are associated with non-disinfected swimming pools [1].

An opportunistic pathogenic bacterium, *Staphylococcus aureus*, is frequently found as member of the microflora of skin or nasal mucosa of healthy individuals and is invariably shed when immersing into the pool water [110]. Its presence in the water in high numbers may be a consequence of crowding and inadequate disinfection and may cause skin infections (rashes, impetigo, otitis externa) wound infections, conjunctivitis, etc. It is sometimes involved in outbreaks (in the above referred ten years, 2 were reported to the CDC WBDOSS team), but it is rather infrequent. Growing concern is attributed to emerging methicillin resistant strains [111]. Density of coagulase positive *staphylococci* in water have been proposed as an indicator with relevance for both the bather load and the effective disinfection [112].

There is only anecdotal evidence of transmission via water of swimming pools or other pool types of *Trichomonas vaginalis*, an unicellular protozoan known as causative agent of trichomoniasis, a rather prevalent sexually transmitted disease. It causes mild, but without treatment lasting nuisance symptoms in infected women, though the proportion of asymptomatic infections is about 70%. The possibility to contract infection this way is controversial. Trichomonas have been shown to survive and remain viable for several hours in swimming pool water [113] but others argue against it claiming that it loses infectivity very quickly in water [114].

A frequently encountered viral skin lesion, *molluscum contagiosum* (sometimes called water wart or swimming pool wart) is caused by *molluscum contagiosum virus* (MCV), a member of the family of the double stranded DNA *Poxviridae*. MCV is infecting only humans and spreads mostly via direct skin-to-skin contact but indirect spread via fomites is also frequently reported. The infection is most prevalent in children under 10 years and is occasionally proposed to be associated to the use of swimming pools [115]. Researchers who have investigated this idea think it is more likely the virus is spread by sharing towels and other items around a pool or sauna than through water [116]. The infection is presented after 2 to 6 week incubation period on the trunk, legs or arms by round lesions of 1-5 millimeter in diameter which are flesh-colored, dome-shaped, and pearly in appearance. An individual lesion lasts generally 6 to 8 weeks, but it can spread from one skin area to others via autoinoculation which considerably may prolong the process up to 6-8 months.

Another relatively innocuous dermal nuisance, *verruca plantaris* or plantar wart is caused by a human *papillomavirus*. Plantar warts are thought to affect 7-12% of the population with higher prevalence in school aged children. A plantar wart is a benign tumour that appears on the sole of the foot and typically resembles a cauliflower, with tiny haemorrhages under the skin in the centre. It may be painful under pressure when walking or standing. Plantar warts are usually acquired via direct physical contact during barefoot activities on surfaces like shower and changing room floors contaminated with infected skin fragments though not necessarily in association with pool and spa facilities [117], and they are not at all transmitted via pool or hot tub waters. Also, it may spread through autoinoculation. The virus is extremely contagious as it is able to survive several months on dry surfaces until encountering a host. The incubation period ranges from 1-6 months; however, latency periods of up to 3 years or more are suspected. Plantar warts are usually self-limiting, but treatment is generally recommended in order to reduce symptoms and to prevent transmission.

Rather prevalent skin infections proposed to be more widespread among pool and spa patrons are some types of dermal mycoses, caused by *Epidermophyton floccosum* and several species of Trichophyton causing superficial infection of the keratinized areas of the body (skin, hair and fingernails). The far most frequent skin infection in association with pool and spa establishments is tinea pedis or by popular label athlete's foot, generally caused by *T. rubrum* or *T. mentagrophytes*. These can survive on a variety of surfaces and objects, like sand, floors, shower stalls, clothing, and hairbrushes) and are transmitted via wet surfaces where people walk barefoot or by contaminated fomites. Athlete's foot causes scaling, flaking, and itching of the affected skin. In more complicated case, blisters and cracked skin occurs, with pain, swelling, and inflammation of the exposed soft tissues and possibly leading to secondary bacterial infection. Tinea pedis most often manifests between the toes but can infect other parts of the body (called then tinea corporis, tinea cruris, etc.). Depending on the species, dermatophytes can survive up to twenty months on skin scales at room temperature. The role of communal bathing places such as indoor swimming pools or other bathes in the spread of tinea pedis has been well established [118].

Swimming pool related dermatomycoses can only be controlled informed hygiene management measures and by concurrent education of people for using pool establishment consciously about the risks of these and the way of evading them. This is also true in association with all other infections mentioned above supplemented with public health efforts to maintain surveillance for early detection of emerging risks and to bring up and support new, effective means of management.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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Health impact of disinfection by-products in swimming pools

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Abstract. This article is focused on the epidemiological evidence on the health impacts related to disinfection by-products (DBPs) in swimming pools, which is a chemical hazard generated as an undesired consequence to reduce the microbial pathogens. Specific DBPs are carcinogenic, fetotoxic and/or irritant to the airways according to experimental studies. Epidemiological evidence shows that swimming in pools during pregnancy is not associated with an increased risk of reproductive outcomes. An epidemiological study suggested an increased risk of bladder cancer with swimming pool attendance, although evidence is inconclusive. A higher prevalence of respiratory symptoms including asthma is found among swimming pool workers and elite swimmers, although the causality of this association is unclear. The body of evidence in children indicates that asthma is not increased by swimming pool attendance. Overall, the available knowledge suggests that the health benefits of swimming outweigh the potential health risks of chemical contamination. However, the positive effects of swimming should be enhanced by minimising potential risks.

Key words: swimming pools, disinfection by-products, epidemiology.

Riassunto *(Impatto sulla salute dei sottoprodotti di disinfezione nelle piscine)*. Questo articolo è incentrato sulle evidenze epidemiologiche riguardanti gli effetti sulla salute associati ai sottoprodotti della disinfezione (DBPs) nelle piscine, la cui presenza è una conseguenza indesiderata dei trattamenti di disinfezione per il controllo dei microrganismi patogeni. Sulla base di studi sperimentali, specifici DBPs sono risultati cancerogeni, fetotossici e/o irritanti delle vie respiratorie. Le evidenze epidemiologiche mostrano che nuotare nelle piscine durante la gravidanza non è associato ad un incremento di rischio di danni riproduttivi. Uno studio epidemiologico ha suggerito un incremento di rischio di cancro alla vescica associato alla frequentazione delle piscine, sebbene le evidenze non permettano di trarre conclusioni certe. Una più alta prevalenza di sintomi respiratori inclusa l'asma è stata trovata tra il personale che opera all'interno delle piscine e i nuotatori ad alta frequentazione, sebbene la causalità di questa associazione non sia chiara. Il peso delle evidenze nei bambini indica che l'asma non aumenta con la frequentazione delle piscine. Complessivamente, le conoscenze attuali suggeriscono che gli effetti benefici del nuotare superano i rischi potenziali per la salute dovuti alla contaminazione chimica. Tuttavia, questi effetti positivi dovrebbero essere aumentati minimizzando i rischi potenziali.

Parole chiave: piscine, sottoprodotti di disinfezione, epidemiologia.

INTRODUCTION

Swimming is a highly practiced sport. There is no question that many positive aspects are associated to swimming in pools, including those from physical activity, back pain relief, and wellbeing from leisure and recreation. In contrast, the World Health Organization identifies 3 hazards in swimming pools: injury/drowning, microbial hazards and chemical hazards [1]. Consequently, eventual positive health effects gained by swimming can be increased by reducing the potential adverse health risks [2]. We will focus this review on the negative impacts related to disinfection by-products (DBPs), which is a chemical hazard generated as an undesired consequence to reduce the microbial hazards.

OCCURRENCE OF DISINFECTION BY-PRODUCTS IN SWIMMING POOLS

Disinfection is necessary in swimming pools to maintain hygienic conditions and avoid water-borne infections. Chlorine is the most widely used disinfectant, that in the presence of organic matter from bathers (urine, sweat, hair, cells, cosmetics, etc.) re-

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acts to generate by-products [2, 3]. Many by-products identified in swimming pools also occur in drinking water [4, 5]. Disinfection by-products occurring at higher concentrations both in drinking water and swimming pools are trihalomethanes (THMs) and haloacetic acids (HAAs). Haloacetoaldehydes, especially trichloroacetaldehyde (chloral hydrate, CH), have also been identified in swimming pools [6]. The presence of nitrogen-containing organic matter from bathers leads to the formation of nitrogenated by-products such as chloramines (mochloramine, dichloramine, trichloramine), haloacetonitriles (HANs) [7] and carcinogenic nitrosamines [8, 9]. Trichloramine is a volatile, irritant compound of penetrating odor, whose main precursors are urea, ammonium ions and α-amino acids [10]. A survey conducted in 86 swimming pools in Seoul, Korea, showed that HAAs occurred at highest concentrations in all swimming pools, regardless of disinfection method, followed by THMs, CH, and HANs [11]. Haloacetic acids also occur in air of swimming pools, in the form or aerosols [12]. Although more than 600 DBPs have been identified, this figure represents less than 50% of total organic halides, and new by-products are to be identified [5].

The formation of DBPs in swimming pools depends on several factors, including the amount of precursors in the water. Levels of THMs in water are correlated with the number of people in the pool, water temperature, and the amount of total organic carbon [13]. Air-water THM concentrations are highly correlated [14, 15], and it has been shown that both THM and trichloramine levels in air increases with the number of swimmers [14, 16, 17] and is also influenced by the volume of total air in the hall [16]. Higher levels have been observed in winter compared to summer, depicting that low air exchange rate in winter lead to accumulation of pollutants [16]. Outdoor compared to indoor pools tend to show higher THM levels in water [18]. Air trichloramine levels are associated with bather loading [19], air temperature and pH [16].

Many by-products of chlorination are genotoxic [4]. However, the mutagenic or genotoxic potency of swimming pool water has been evaluated in very few studies. Mutagenicity was assessed by Richardson *et al.* [5] in samples collected in Barcelona, Spain. The authors concluded that swimming pool waters were as mutagenic as typical drinking waters [5]. Genotoxicity of water concentrates from recreational pools after various disinfection methods was evaluated by Liviac *et al.* [20], showing that all disinfected recreational pool water samples induced more genomic DNA damage than the source tap water. They also found that the type of disinfectant and illumination conditions altered the genotoxicity of the water. A previous study evaluated genotoxicity of swimming pool water, observing strongest potencies in the low-molecular weight fraction [21]. The implications are that removal of DBPs with higher genotoxic potential requires membrane treatment with low-molecular weight cut offs, which is an advanced treatment not generally used in swimming pools. Later, it has been shown that pool water quality could be improved with nano- and ultrafiltration, compared to the conventional treatment [22].

Alternative disinfectants used in swimming pools include advanced processes like ozonation, copper-silver ionization, UV radiation, among others. Some of them (*e.g.* ozonation) are used in combination with chlorination. The formation of chlorination by-products are reduced by the use of these alternative disinfection methods, as illustrated by a study conducted in Seoul, Korea, where THMs, HAAs, HANs and CH levels were lower in chlorine/ozone swimming pools compared to only chlorinated pools [11]. However, these alternative disinfection processes still involve the formation of by-products of concern [23, 24]. For example, bromate is generated by ozonation if bromine is present in the water [25]. Bromine-based disinfectants are also used in some swimming pools and spa, where brominated DBPs are produced in highest concentrations [15].

Disinfection by-products are regulated in drinking water in most western countries, where trihalomethanes and bromate are routinely measured [26], as well as haloacetic acids in the US [27]. The World Health Organization suggests that trichloramine should be monitored in swimming pools and the recommended guideline is set at 0.5 mg/m^3 [1]. However, this recommendation is not included in the local regulations for swimming pools, and DBPs are not controlled in swimming pools on a regular basis.

HUMAN EXPOSURE

Exposure to DBPs in swimming pools occurs mainly through inhalation of volatile DBPs or aerosol containing DBPs and through dermal absorption of skin-permeable DBPs. Volatile DBPs include THMs and trichloramine, among many others [7, 28]. Swimming pool attendance represents a relevant contribution in the total burden of THM exposure, especially when subjects drink bottled water [29].

Human exposure to THMs in swimming pools has been evaluated in several studies that measured THMs in blood, exhaled air and urine. While the four THMs (chloroform, bromodichloromethane, dibromochloromethane and bromoform) can be measured in exhaled air, only chloroform is usually detected in blood [30, 31]. Chloroform and bromodichloromethane are the only THMs detected in urine [32]. In consequence, it has been suggested that alveolar air would be the most sensitive exposure biomarker [33], particularly at low environmental levels [34]. Level of physical activity is correlated with internal dose [15, 17, 31, 35, 36]. The relative importance of inhalation vs. dermal absorption has been evaluated for chloroform. Lévesque *et al.* [37] dissociated the dermal from the inhalation exposure routes in 11 male swimmers and measured alveolar chloroform levels, concluding that approximately

24% of body burden resulted from dermal absorption. Erdinger *et al.* [38] found that blood levels were highly correlated with air levels and poorly correlated with water concentrations, suggesting that that inhalation would be more important than dermal absorption and estimated that one third of the total burden is taken up over the skin. On the contrary, Lindstrom *et at*. [39] estimated that dermal exposure accounted for 80% of the blood concentration, based on alveolar air measurements in 2 elite swimmers.

Although HAAs are non-volatile and have little skin permeability, swimmers may be exposed through inhalation of aerosol [12]. Internal dose of HAAs in 49 swimmers and pool workers has been measured in urine by Cardador *et al.* [40]. Dichloroacetic (DCAA) and trichloroacetic (TCAA) acids were detected in urine among workers after two hours of exposure. After 1 h swimming, TCAA, DCAA and monochloracetic acid (MCAA) were detected in swimmers at concentrations much higher than those measured in workers. Similar results were obtained from swimmers in an outdoor pool due to accidental ingestion. The authors estimate that ingestion is the major route of exposure (94%), followed by inhalation (5%) and dermal contribution (1%). Personal exposure assessment using biomarkers has not been conducted for other DBPs.

RESPIRATORY HEALTH

First evidence that swimming pool environment can be toxic for respiratory health come from case reports of acute intoxication in swimming pools due to accidental chlorine exposures [41-44]. Symptoms described by these cases vary according to the level of exposure and include eye and airway irritation, cough, wheezing and shortness of breath, and in worst cases, respiratory impairment requiring mechanical ventilation [45, 46]. Epidemiological studies have mainly been focused on three specific populations: pool workers, professional swimmers and children. Workers and elite swimmers are highly exposed populations, and children are particularly vulnerable to environmental insults.

Occupational exposure

Swimming pools are the occupational environment of lifeguards, swimming trainers and professional swimmers. This chronic occupational exposure has been studied in relation to respiratory symptoms. Massin *et al.* described in France that trichloramine levels in swimming pools' air were associated with the prevalence of eye and nasal irritant symptoms among 334 lifeguards working in the pools, but not with the prevalence of chronic respiratory symptoms such as bronchial hyperreactivity [47]. Jacobs *et al.* also found that among 624 swimming pool workers in The Netherlands, lifeguards and swimming trainers had higher prevalence of nasal symptoms but not bronchial hyperreactivity than other workers not directly exposed to the swimming pool environment [48]. In Italy, a study among 115 swimming pool workers found that those exposed to higher DBP levels (measured with THM in exhaled breath) had higher prevalence of asthma, although only 10 asthmatics were included [49]. In a water park in US, lifeguards had higher prevalence of cough, shortness of breath and wheezing than workers not directly exposed to the pool environment [50]. Due to the cross-sectional design of these studies, a healthy worker effect could have occurred in the studies; *i.e.*, self-selection by workers, with sensitive subjects quitting or avoiding this job. On the other hand, Thickett, *et al.* described 3 cases of occupational asthma due to trichloramine sensitization in swimming pool lifeguards [51].

Elite swimmers

Different studies have described higher prevalence of asthma or respiratory symptoms among swimmers than among other competitive athletes [46, 52, 53]. In 2008, Goodman and Hays published a meta-analysis including 6 studies and found a higher prevalence of asthma among swimmers compared to other elite athletes (OR = 2.57; 95% CI = 1.87-3.54) [54]. The authors stated that asthmatics are more frequently found among swimmers but that the cause of this association is not clear. The chronic exposure to the irritant environment could lead to an increased airway inflammation and more asthma symptoms among swimmers [55]. On the other hand, asthmatics may be more prone to choose swimming compared to other sports and therefore more asthmatics are found among swimmers. Swimming is one of the less asthmagenic and more safe sports to practice for asthmatic patients [56, 57] and it is the recommended sport for them [58]. In 72 young competitive swimmers, Levesque *et al.* found that those exposed to higher levels of trichloramine in air had higher prevalence of upper respiratory symptoms [52]. Recent studies have described also higher prevalence of asthma symptoms among competitive swimmers than among the general population [59, 60]. In summary, swimmers have higher prevalence of asthma compared to other athletes. However, the direction of the association is not clear and both chemical exposure and the preference of swimming among asthmatics may be responsible for the detected association [53-55].

Children

The possibility that the exposure to the swimming pool environment can affect respiratory health of recreational swimmers and especially among children has raised concern in recent years [55, 61-69]. If a causal association were detected, public health implications would be relevant [63]. On one hand, asthma is among the most common chronic diseases in children [70], with an increasing incidence, prevalence, morbidity, mortality and economic cost in recent decades [71]. On the other hand, swimming

is one of the most practiced sports in western countries [72], where sedentarism and obesity are increasing, especially among children [73].

First studies were conducted in Belgium [74-76], Germany [77, 78] and Italy [79]. The studies in Belgium found an increased risk of childhood asthma among atopic children related to both indoor and outdoor pool attendance, while the other studies did not. A metanalysis including these studies showed that the OR of having asthma was not increased with pool attendance among children [54]. However, several shortcomings were identified in the exposure assessment and the characterization of asthma [63]. Since then, two studies have been published in Belgium [80, 81]. Among 430 children at 6 years of age, an increased risk of bronchiolitis, but no wheezing or other respiratory symptoms, was found among those with higher pool attendance [81]. An increased risk of asthma was also found among adolescents with higher chlorinated pool attendance [80]. A small study in Ireland [82] found a significant association between asthma and the number of years attending pools among adolescents, but not with the frequency of attendance. A larger Spanish study found a null association among children 10 to 12 years old [83]. All epidemiological studies until that moment used a cross-sectional design with retrospective assessment of swimming pool attendance, which could have introduced recall bias, exposure misclassification and reverse causation [55, 62, 63].

The first longitudinal prospective study used data from the ALSPAC child cohort in UK [84]. The study analysed 5738 children with data on swimming pool attendance collected at different time points during childhood and with data on respiratory health assessed with questionnaires and spirometry. The results indicated that swimming did not increase the risk of asthma, atopy or any respiratory and allergic symptom in children at 7 or 10 years of age [84]. On the contrary, swimming was associated with increased lung function and with a decreased prevalence of current asthma among children with previous respiratory conditions. This occurred regardless of the socioeconomic status of the family. This positive association between swimming and lung function could be explained by the benefits of physical activity on the respiratory health. In fact, there is growing evidence that physical activity during childhood [77, 85, 86] or during adulthood [87] may decrease prevalence of allergic rhinitis [77], bronchial hyper reactivity [87] and asthma development [85, 86]. If the results detected in the ALSPAC cohort are further confirmed, swimming would not only be a safe sport for people with asthma [56] but also may help control asthma symptoms [84]. Other recent studies also suggest that people with asthma may improve their asthma symptoms with swimming [54, 56, 88, 89].

The inconsistency of the results among studies may reflect true differences in the association between swimming and childhood asthma (due to different patterns of pool attendance or different level of irritants in the swimming pools) or may relate to methodological aspects. Only a few epidemiological studies have reported trichloramine levels [75, 80, 83] and overall levels were below the 0.5 mg/m^3 recommended by the World Health Organization [1]. Concerning the methodological differences, the studies with null associations [77, 78, 83, 84] are based on large and population-based samples, whereas the studies with positive results are based on smaller datasets [74-76; 80-82]. Epidemiological studies on the topic are complex and several limitations still have to be overcome in future studies. If an association between swimming and asthma in childhood exists, the direction of the association, and therefore its causality, should be clarified. On one hand, a healthy swimmer effect may occur (*i.e.* children with asthma abandon swimming and therefore healthier children are found among studied swimmers) but also the other may occur since swimming is a recommended sport for asthmatics. This selection bias is not directly solved in a longitudinal study and should be carefully addressed in the studies. Exposure assessment should be also improved with more data on the levels of potential irritants in swimming-pool's air and with validated questions on swimming pool attendance. Furthermore, swimming in pools entails two kinds of exposures with an impact on the respiratory health: chemical (or biological) exposure and physical activity. Finally, future studies should measure potential confounding more carefully, especially other forms of physical activity.

Baby swimming, *i.e.* swimming pool attendance during the first year of life, has become a popular activity in many countries. This is a vulnerable period of life, and some studies have evaluated the effect of this activity on the subsequent respiratory health of the children. Overall, no increased risk of wheezing or respiratory infections has been observed, but inconsistent results have been described among baby swimmers with atopic mothers [90, 91].

Although further studies are still needed, most of the scientific evidence does not support the hypothesis that recreational swimming increases the risk of childhood asthma. The benefits of swimming (prevention of obesity, diabetes, etc) seem to largely outweigh the potential risks of chemical contamination, as concluded by the Superior Health Council of the Belgian Government [92]. Based on the precautionary principle, this report by the Belgian Government does not encourage swimming in chlorinated pools during the first year of life especially among babies with atopic or asthmatic parents. They argue that the health benefits of baby swimming are less pronounced that swimming after being 3 or 4 and these benefits may be acquired with other activities.

CANCER AND ADVERSE REPRODUCTIVE OUTCOMES

Epidemiologic evidence indicates that exposure to THM, the most concentrated DBP, relates to

an increased risk of bladder cancer [93, 94]. Only one study have assessed specifically this exposure through swimming pools and found increased bladder cancer risk among subjects who had attended swimming pools [95]. Nelemans, *et al.* [96] observed a positive association between a history of swimming and melanoma risk, suggesting that carcinogenic agents in water, possibly chlorination by products, play a role in melanoma aetiology. Evidence for an increased risk of other types of cancers have not been evaluated in relation to exposure through swimming pools. Other health risks that have been studied in relation to DBP exposure are adverse reproductive outcomes. Small DBP molecules can pass from the mother to the foetus through the placenta [97, 98]. Evidence from animal experiments shows a range of adverse reproductive effects, including sperm toxicity, teratogenicity, and reduced fetal growth [99]. Epidemiological studies reported inconsistent results [99, 100], but recent reviews and meta-analysis indicate little or no evidence that THM exposure through different routes during pregnancy is associated to fetal growth [101] and congenital anomalies [102]. Specific studies on exposure through swimming pools overall also indicate no effect on adverse birth outcomes [103-105]. A recent study has described lower testicular hormone levels among Belgian adolescents having attended indoor chlorinated pools [106].

SHORT-TERM MOLECULAR CHANGES IN HEALTHY ADULTS: THE PISCINA STUDY

In order to provide evidence on mechanisms of action of DBP exposure, an experimental study in 50 healthy adult volunteers was conducted to evaluate a battery of biomarkers of genotoxicity and lung damage after swimming in a chlorinated pool.

General study design

An experimental study consisting of a before-after comparison was conducted in Barcelona, Spain, in 2007. Fifty non-smoking adult volunteers, after signing informed consent, swam for 40 minutes in a chlorinated pool. THM levels were determined in pool air and water, water mutagenicity, THM levels in exhaled air, micronuclei and the comet assay in peripheral blood lymphocytes and urine mutagenicity. Blood and exhaled breath condensate (EBC) were collected and lung function and FENO were measured before and after the exposure event. CC16 and SP-D were measured in serum and 8-isoprostane and cytokines in EBC. Urine samples were collected before and 2 weeks after the swimming session. Chronic respiratory symptoms, usual swimming habits, water use, diet and lifestyle factors were collected through an extensive questionnaire. In addition, gas chromatography (GC)/mass spectrometry (MS) was used to comprehensively identify DBPs in pool waters. More detailed methods have been published [5, 107, 108].

Exposure

The mean level of free chlorine in pool water (1.28 mg/L) was similar to that found typically in drinking water [5]. More than 100 DBPs were comprehensively identified in the pool waters and all contained either bromine or chlorine and no iodinated DBPs were detected. Most DBPs had not been reported previously for swimming pool waters [5]. Total THM levels in pool water were around 50 µg/L and 72 µg/m^3 in air [5]. After swimming, THMs in exhaled breath of swimmers increased on average about seven-fold. No differences were observed by age group, sex, or body mass index [107]. The measured THM concentrations in exhaled breath constitute about one eighth of the ambient indoor concentrations [15]. Chloroform levels in exhaled breath were significantly correlated with levels in the swimming pool air, but not with water levels. Dichloramine in water was inversely and significantly correlated with brominated THMs but not with chloroform in water, air, and exhaled breath. Free chlorine in water was not significantly correlated to total THMs in water but was significantly correlated to total THMs in air and exhaled breath. The energy expenditure of volunteers correlated significantly only with bromoform concentration in exhaled breath after swimming [107]. Trichloramine in water was undetectable and trichloramine in the air ranged from 0.17 to 0.43 mg/m^3 (mean, 0.29 mg/m^3), which is below the World Health Organization recommendations of 0.5 mg/m^3 [5].

Respiratory outcomes

Molecular markers measured included biomarkers of airway inflammation (exhaled nitric oxide -FENO- and cytokines in EBC), oxidative stress (8-isoprostane), lung permeability and immunological response (surfactant protein –SP-D) and lung damage (Clara cell protein –CC16-). We detected no significant changes in lung function (percent predicted FEV1, FVC, FEV1/FVC), airway inflammation (measured with FeNO and cytokines in EBC) or oxidative stress (measured with 8-isoprostane in EBC). The concentration of CC16 in serum, which is a marker of lung epithelium permeability, was increased slightly but significantly after swimming 40 minutes, with an overall median increase of 0.47 µg/L (3.3% increase) [107]. The increase in serum CC16 concentration was significantly correlated with different indicators of DBP exposure (negatively with dichloramine in water and positively with free chlorine in water and bromodichloromethane, dibromochloromethane, and bromoform in exhaled breath) and with energy expenditure, an indicator of physical activity. In multivariate models, both energy expenditure and markers of DBP exposure remained significantly associated with the increase in CC16 after mutual adjustment suggesting that these exposures may contribute to the change in lung permeability [107]. However, given the moderate increase detected, the high variability in CC16

levels in healthy subjects [109] and the lack of reference values of CC16, the clinical relevance of this short-term effect is unclear [110] and further studies are necessary to establish the health impacts of short-term serum CC16 changes [109, 111].

Genotoxicity

The comet assay was conducted in peripheral blood lymphocytes (PBLs). One hundred cells selected randomly (50 cells from each of the two replicate slides) were analyzed per sample. Olive tail moment (OTM) and percentage of DNA in the tail, used as measures of DNA damage, were computed using Komet software, version 5.5 (Kinetic Imaging, Liverpool, UK). The micronuclei (MN) assay was conducted in PBL and urothelial cells. To determine the frequency of binucleated cells with MN and the total number of MN, a total of 1000 binucleated cells with well-preserved cytoplasm (500/replicate) were scored for each subject. In addition, we scored 500 lymphocytes to evaluate the percentage of cells with one to four nuclei and calculated the cytokinesis block proliferation index. The frequency of urothelial cells with MN and the total number of MN were determined for each analyzed subject. We used generalized estimating equations (GEE) to assess associations between THM exposure and changes in genotoxicity markers before and after swimming [108]. A statistically significant increase was found for PBL MN in relation to changes in total THMs in exhaled air. This increase was more pronounced for brominated compounds and was not statistically significant for chloroform. No changes were found for the comet assay. We found no significant associations with changes in micronucleated urothelial cells. Adjustment for several potentially confounding factors did not modify results [108].

OVERALL CONCLUSIONS

The health effects that have been evaluated in relation to DBP exposure in swimming pools include adverse reproductive outcomes, carcinogenicity and respiratory outcomes. Epidemiological evidence does not support an increased risk of reproductive outcomes after swimming in pools during pregnancy. However, a study suggested an increased risk of bladder cancer with swimming pool attendance, although results are far from being conclusive. Extensive research has been conducted to evaluate respiratory outcomes associated with swimming pool attendance. Evidence indicates that the association between asthma risk and swimming depends on the target population [54]. A higher prevalence of respiratory symptoms including asthma is found among those occupationally exposed to the pool environment, although the causality of this association is uncertain. Studies in children are less conclusive but the only longitudinal and prospective study indicates that asthma is not increased by swimming pool attendance [84]. Overall, the available evidence supports that the health benefits of swimming during childhood and adulthood outweigh the potential health risks of chemical contamination [92]. However, the positive effects of swimming should be increased by minimising potential risks. Given the high public health relevance of the topic and the existing uncertainties, further research is needed to draw final conclusions about the risk of respiratory problems during childhood for swimming in well maintained swimming pools. The maximum level proposed for trichloramine in air as provisional guideline [1] should be revised [112, 113] and regulated. Also, the potential carcinogenicity of this environment should be further explored. Efforts should be done to identify which specific DBPs are responsible for the toxicity, to be monitored DBPs in swimming pools [92].

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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Emerging and potentially emerging viruses in water environments

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Abstract. Among microorganisms, viruses are best fit to become emerging pathogens since they are able to adapt not only by mutation but also through recombination and reassortment and can thus become able to infect new hosts and to adjust to new environments. Enteric viruses are among the commonest and most hazardous waterborne pathogens, causing both sporadic and outbreak-related illness. The main health effect associated with enteric viruses is gastrointestinal illness, but they can also cause respiratory symptoms, conjunctivitis, hepatitis, central nervous system infections, and chronic diseases. Non-enteric viruses, such as respiratory and epitheliotrophic viruses are not considered waterborne, as they are not readily transmitted to water sources from infected individuals. The present review will focus on viral pathogens shown to be transmitted through water. It will also provide an overview of viruses that had not been a concern for waterborne transmission in the past, but that may represent potentially emerging waterborne pathogens due to their occurrence and persistence in water environments.

Key words: viruses, waterborne, emerging, drinking water, recreational water.

Riassunto *(Infezioni emergenti e potenzialmente emergenti in ambienti acquatici)*. I virus sono tra i principali agenti di infezioni emergenti, in quanto sono in grado di acquisire nuove caratteristiche biologiche attraverso fenomeni di mutazione, ricombinazione e riassortimento genico, adattandosi così a nuovi ospiti e nuove nicchie ecologiche. I virus enterici sono tra le principali cause di malattie infettive di origine idrica, che possono manifestarsi come casi sporadici o cluster epidemici. Le manifestazioni cliniche più comuni sono le gastroenteriti; tuttavia i virus enterici sono in grado di causare anche sintomi respiratori, congiuntiviti, epatiti, infezioni del sistema nervoso centrale e malattie croniche. La presente rassegna fornisce una panoramica dei virus enterici tradizionalmente associati alle malattie idrotrasmesse; mostra inoltre dati epidemiologici su alcuni gruppi di virus che non sono stati associati a malattie idrodiffuse in passato (inclusi virus respiratori ed epiteliotropici), ma che possono rappresentare nuovi patogeni emergenti a causa della loro presenza e persistenza in ambienti acquatici.

Parole chiave: virus, malattie idrotrasmesse, patogeni emergenti, acque potabili, acque ricreative.

INTRODUCTION

The human illnesses associated with enteric viruses are diverse and their severity varies. The symptoms most commonly associated with enteric viral infections are those of gastroenteritis (GE). Some enteric viruses, however, are responsible for respiratory symptoms, conjunctivitis, hepatitis, central nervous system infections (aseptic meningitis, poliomyelitis) and muscular syndromes (fibromyalgia, myocarditis). Enteric viruses have also been implicated in some chronic diseases, including diabetes and chronic fatigue syndrome. Patients suffering from viral infections may excrete 10^5 to 10^{11} virus particles per gram of stool. Consequently virus concentrations in wastewaters receiving fecal matter are high. The degree of wastewater inflow contamination depends on the season, the prevalence of viral infections and the characteristics of circulating viruses. Wastewater treatment systems, even when properly functioning, remove only about 20-80% of enteric viruses [1-6], allowing a significant viral load to be released in effluent discharge and spread in the environment, transported through groundwater, estuarine water, seawater and rivers. The concentration of enteric viruses in water can vary significantly in time and space, depending also on whether the source of pollution is continuous or the result of a sudden influx of fecal contamination.

Viruses cannot replicate outside their host's tissues and therefore cannot multiply in the environment. They can, however, survive in the environment for extended periods of time, longer than most intestinal bacteria, making it unsafe to rely solely on bacteriological water quality standards. Viruses have been reported to survive and remain infective for up to 130 days in seawater, for up to 120 days in freshwater, and for up to 100 days in soil at 20-30 °C [7]. Comparisons between enteric viruses also show variability, with adenoviruses potential-

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ly surviving longer in water than other enteric viruses, such as hepatitis A virus and poliovirus [8]. Despite the relatively low concentration of viruses in water, these micro-organisms carry health risks, since they have very low infectious doses (10-100 virions) so that even a few viral particles in water can pose health risks.

The global impact of waterborne disease is difficult to assess. This is due to the wide range of clinical manifestations associated with waterborne viruses, to potentially long latency periods, and to the fact that in some cases, diseases related to water can also be transmitted in other ways. Moreover, evidence of water contamination is not always available by the time an outbreak of disease is identified, mainly due to the difficulties associated with the detection of viruses in water. Indeed, viral detection in water environments presents particular challenges due to low pathogen concentrations, and the multitude of enteric viruses requiring different analytical methods. Data on viral waterborne diseases is fragmented, often focusing on specific countries and/or pathogens. In US, the Centre for Disease Control (CDC), the Environmental Protection Agency, and the Council of State and Territorial Epidemiologists established the Waterborne Disease and Outbreak Surveillance System (WBDOSS) to collect and report data related to occurrences and causes of waterborne disease outbreaks associated with drinking and recreational waters. Data from the WBDOSS drinking water surveillance from 1971 (a 36-year period) were recently reviewed by Craun and collaborators [9]: an etiology was identified only in 56.1% of the outbreaks reported (7.9% of viral origin). Since viral outbreaks linked to recreational or drinking waters are often underestimated [10, 11] enteric viruses could be responsible for a significant portion of outbreaks of "undetermined" etiology. According to the last WBDOSS report, during the 2007-2008 surveillance period, 13.9% of drinking and 4.8% of recreational water-associated outbreaks were caused by viruses [12, 13]. The number of outbreaks of unknown etiology was 11.1% for drinking and 21.6% for recreational waters, thus indicating a decrease of "unidentified" etiologies in recent years, probably due to the availability of improved methods for the detection of waterborne pathogens.

Emerging waterborne diseases can be defined as those that have newly appeared, or are rapidly increasing in incidence and/or geographic range, or those for which water transmission routes have only recently been recognized. Complex relations between the pathogen, the host, and the natural environment determine the emergence of pathogens. In the globalized world, the circulation of microbes are facilitated so that infectious agents are capable of spreading rapidly anywhere in the world. Among microorganisms, viruses are best fit to become emerging pathogens since they are able to adapt not only by mutation but also through recombination and reassortment and can thus become able to infect new hosts and to adapt to new environments. Emerging waterborne enteric viruses belong to the families *Caliciviridae* (norovirus), *Picornaviridae* (enterovirus and hepatitis A virus) and *Adenoviridae* (adenovirus). These pathogens are included in the Environmental Protection Agency Contaminant Candidate List 4 - CCL, a list of contaminants that are currently not subject to any proposed or promulgated primary drinking water regulations in the USA, but are known or anticipated to occur in public water systems. All these viral pathogens have been detected in sewage, surface water, groundwater and drinking water sources around the world. Other virus groups are considered to be potentially emerging waterborne pathogens and include hepatitis E virus, the viral agent of avian influenza, coronavirus, polyomavirus, picobirnavirus, and papillomavirus.

The present review will focus on viral pathogens shown to be transmitted through water. It will also provide an overview of viruses that had not been a concern for waterborne transmission in the past, but that may represent potentially emerging waterborne pathogens due to their occurrence and persistence in water environments. *Table 1* shows human viruses described in this review, that are potentially transmitted by the waterborne route.

Table 1 | *Human viruses potentially transmitted by the waterborne route.*

Virus group	Genus	Family	Disease caused
Norovirus	Norovirus	*Caliciviridae*	Gastroenteritis
Human enterovirus A-D	Enterovirus	*Picornaviridae*	Paralysis, herpangina, meningitis, respiratory disease, hand-foot-and-mouth disease, myocarditis, heart anomalies, rush, pleurodynia, diabetes
Hepatitis A virus	Hepatovirus	*Picornaviridae*	Hepatitis
Human adenovirus A-G	Mastadenovirus	*Adenoviridae*	Gastroenteritis, respiratory disease, conjunctivitis
Hepatitis E virus	Hepevirus	*Hepeviridae*	Hepatitis
Influenza A virus	Influenza A virus	*Orthomyxoviridae*	Influenza
Human coronavirus	Coronavirus	*Coronaviridae*	Gastroenteritis, respiratory disease
Human polyomavirus	Polyomavirus	*Polyomaviridae*	Skin diseases, progressive multifocal leukoencephalopathy, nephropathy, hemorrhagic cystitis
Human picobirnavirus	Picobirnavirus	*Picobirnaviridae*	Diarrhea
Papillomavirus	Papillomavirus	*Papillomaviridae*	Genital warts, throat warts (rarely), skin warts, cervical cancer and other, less common but serious cancers

VIRAL PATHOGENS TRANSMITTED THROUGH WATER

Norovirus

Noroviruses (NoV) are non-enveloped, single-stranded RNA viruses, belonging to the family *Caliciviridae*, currently subdivided into five genogroups (GI, GII, GIII, GIV, GV), comprising at least 40 genetic clusters, subdivided into various types. Genotypes infecting humans are those belonging to GI, GII and GIV. Human NoV is emerging as the leading cause of epidemic GE and as an important cause of sporadic GE in both children and adults worldwide. It is responsible for nearly half of all GE cases and for more than 90% of non-bacterial GE epidemics worldwide [14, 15]. NoV infection induces vomiting, diarrhea, mild fever, abdominal cramping, and nausea. Although typically with a self-limiting disease of short duration, new evidence suggests that the illness can be severe and sometimes fatal, especially among vulnerable populations -young children, the elderly and the immunocompromised- and is a common cause of hospitalization. Numerous reports have associated NoV with clinical outcomes other than GE, such as encephalopathy, disseminated intravascular coagulation, convulsions, necrotizing enterocolitis, post-infectious irritable bowel syndrome, and infantile seizures. Outbreaks have often been reported from institutional settings (*e.g.*, nursing homes, hospitals, day care, cruise ships), where NoV can be especially difficult to control due to efficient person-to-person transmission, and virus resistance to common cleaning agents.

In Europe and the United States, NoV epidemics have been reported to increase in both incidence and severity, probably as a result of an increased pathogenicity and/or transmissibility of new strains [16, 17]. The availability of improved detection techniques, combined with an enhanced awareness to the potential risks associated with this pathogen, has led to the establishment of several surveillance systems, national (Calicinet in the US, Vironet in Canada, the Norovirus Surveillance Network in Australia and New Zealand) as well as global (Noronet). The Foodborne Viruses in Europe (FBVE) network has been collecting laboratory and epidemiological data on NoV outbreaks in Europe since 1999.

The primary mode of transmission is faecal-oral and occurs through the ingestion of contaminated water, consumption of contaminated food, or direct contact with environmental surfaces or infected persons. Numerous outbreaks have originated from sewage-polluted drinking and recreational water. Norovirus belonging to GI are significantly more likely to have been transmitted via water than by other routes of transmission, and are therefore thought to be more stable in water than GII strains. Noroviruses have been detected in a wide range of water environments such as sewages, municipal water, rivers, recreational waters, and groundwater throughout the world [18-30].

Noroviruses are highly resistant to adverse environmental conditions. They can survive chlorination in concentrations up to 10 ppm and temperatures ranging from below 0 °C to 60 °C and higher. The NoV genome may persist for 1-3 months in different types of water (mineral, tap water and river) [31]; GI NoV stored in groundwater was still infectious in human volunteers after 2 months, and GI NoV RNA stored in groundwater was still detectable by RT-PCR after 588 days [32, 33].

Several reviews on waterborne NoV outbreaks have been published [10, 20, 34]. The most recent paper reviewed all publications on the PubMed database reporting waterborne NoV outbreaks from the Norwalk epidemic that led to the discovery of the virus in 1969 up to the year 2007. In the 38 years covered, the review found 43 waterborne NoV outbreaks in various parts of the world (involving NoV alone or together with other enteric viruses) [20]. In the four years between 2008 and the present, on the other hand, a similar review found 24 reported outbreaks (La Rosa, unpublished data) associated with drinking water from private wells, community water systems, fountains, and surface water such as lakes and swimming pools. NoV are believed to be the major cause of recreational waterborne outbreaks (45%, followed by adenovirus 24%), documented in the published literature [11].

The explosion of data on NoV outbreaks in recent years is reason for concern. It is, however, unclear whether this is the result of improved reporting, surveillance and detection, or an actual increase in incidence.

Enterovirus

Human enteroviruses, members of the family *Picornaviridae*, consist of non-enveloped virus particles containing a 7,500-nucleotide single-stranded RNA genome protected by an icosahedral capsid. They comprise more than 100 serotypes, which are currently grouped into four species, Human enterovirus A-D, based on molecular and biological characteristics [35]. The health effects associated with enterovirus infections are varied, ranging in severity from mild to life threatening. Mild symptoms include fever, malaise, sore throat, vomiting, rash and upper respiratory tract illnesses, acute gastroenteritis (less common). Viraemia can occur, transporting enteroviruses to various target organs and resulting in potentially serious complications such as meningitis, encephalitis, poliomyelitis and myocarditis. Other complications include myalgia, Guillain-Barré syndrome, hepatitis and conjunctivitis. Enteroviruses have also been implicated in the aetiology of chronic diseases, such as inflammatory myositis, dilated cardiomyopathy, amyotrophic lateral sclerosis, chronic fatigue syndrome, and diabetes mellitus. Since these viruses are common, are shed in extremely high numbers from infected individuals, and are stable in the environment for extended periods of time, they have been suggested as a parameter for evaluating viral pollution of environmental waters [36, 37]. Enteroviruses are resilient organisms, able to survive drastic changes in temperature and pH [6, 38]. Enteroviruses have been detected in both raw and treated sewages [5, 39-44]. Studies on removal efficiencies at wastewater treatment plants, based on real-time PCR, showed that enteroviruses are more resistant to treatment than other enteric viruses (adenoviruses and noroviruses) [5]. Moreover, live virions have been detect-

ed in treated waters through infectivity assays, pointing to potential public health risks [5, 40]. The occurrence of enteroviruses in coastal waters in both bathing and non-bathing sites is documented worldwide [45-50]. Enteroviruses have also been found in rivers [49, 51-55], lakes [56, 57], groundwater [29, 58-61] and in both untreated and finished drinking water supplies [30, 38, 55, 62, 63]. Despite the widespread presence of these viruses, however, few drinking water-related outbreaks have been reported [64-67], only two outbreaks of recreational waterborne coxsackievirus, nine echovirus outbreaks concentrated between 1990 and 2005 [11] and sporadic echovirus 30 outbreaks in swimming pool. The limited knowledge on the role of waterborne enterovirus transmission could be related to a number of factors, including the wide range of clinical symptoms, frequent asymptomatic infection, the diversity of serotypes and the dominance of person-to-person spread.

Hepatitis A virus

Among the types of hepatitis viruses identified so far, only two types, hepatitis A (HAV) and hepatitis E (HEV) are transmitted via the faecal-oral route and therefore associated with waterborne transmission. HAV is an icosahedral non-enveloped, single-stranded RNA virus belonging to the *Picornaviridae* family, genus Hepatovirus. HAV infections result in numerous symptoms, including fever, malaise, anorexia, nausea and abdominal discomfort, followed by jaundice; the infection can also cause liver damage. Fulminant hepatitis is a rare complication, posing an increased risk to elderly patients and to patients with chronic liver disease. The incidence of infection varies between regions of the world, with the highest rate in non-industrialized countries where sewage treatment and hygiene practices can be poor. Conversely, the number of reported cases of HAV infection has declined substantially in countries with effective immunization programs. The virus is excreted in the feces (and urine) of infected persons and can contaminate soil, water (fresh or seawater) and food, including shellfish (mussels and oysters) harvested from contaminated water. The virus can survive for up to 60 days in tap water, over 6 weeks in river water, over 8 weeks in groundwater and even up to 30 weeks in sea water [68]. HAVs have been detected in different water environments: wastewaters [69-74], treated effluents [75], surface waters [51, 76-80], and drinking waters [81-83]. Outbreaks of hepatitis A among persons who use small private or community wells have been reported [84-87]. According to a review based on WBDOSS drinking water surveillance data spanning a 36-year period, 45.3% of the 64 viral drinking water outbreaks were attributed to hepatitis A virus [9]. As for recreational waterborne hepatitis A, four outbreaks were identified, linked to lakes, pool, thermal pool/spa [11]. The morbidity contributed by these four outbreaks is low when compared to outbreaks of other etiologies.

Adenovirus

Human adenoviruses (HAdVs) are non-enveloped, icosahedral viruses of the genus Mastadenovirus, family *Adenoviridae*. These viruses have a linear, double stranded DNA genome (26-45 kb) encapsidated in an icosahedral protein shell. There are 51 serotypes classified into six species, A–F, defined using biological characteristics. Additional types continue to be identified and characterized using genomics and bioinformatics. Clinical manifestations are highly heterogeneous, ranging from upper and lower respiratory tract infections to gastroenteritis, pneumonia, urinary tract infection, conjunctivitis, hepatitis, myocarditis and encephalitis [88, 89]. Adenoviruses can cause severe or life-threatening illness, particularly in immunocompromised patients, children and the elderly. Adenoviruses have been widely detected in wastewaters (both influent and effluent sewages) worldwide [88]. Different studies reported AdV concentrations to be the highest of all enteric viruses tested in these matrices [5, 90, 91]. The viral load in the feces of infected individuals is high (~10^6 particles/g of fecal matter). In addition, AdVs are very resistant to UV light and may survive longer than fecal indicator bacteria both in sewage and in the environment. Adenoviruses have also been frequently detected in surface waters. In comparative studies, AdVs usually outnumbered enteroviruses in surface waters [88]. A Europe-wide surveillance study carried out to determine the frequency of AdV and NoV in recreational waters (marine and freshwater samples), detected AdVs in 36.4% of samples, of which approximately 25% were infectious, supporting the case for considering AdVs a good indicator of bathing water quality [27]. Quantitative data on AdV in these waters showed mean values of 3.2 x 10^2 genome copies per 100 ml of water [92]. Infectious AdVs have been detected in conventionally treated and disinfected drinking water in Africa and Asia, using genome detection with PCR in cell culture. Limited data suggest that AdVs survive longer in water than either enteroviruses or HAV. They are capable of surviving for months in water, especially at low temperatures. Their increased stability in the environment may be in part attributable to their double-stranded DNA, and/or to their ability to repair damaged DNA by activating cell repair enzymes. This may also enhance their resistance to inactivation by UV light. AdVs have been implicated in drinking water outbreaks, although they were not the only pathogens involved [93, 94]. As for recreational outbreaks, 14 conjunctivitis or pharyngoconjunctival fever outbreaks, linked to pool, lake and pond, have been described, spanning six decades, making AdV the second etiological agent, after NoV [11, 95] for such epidemics.

Hepatitis E virus

Hepatitis E virus (HEV) infections are caused by a positive-sense, non-enveloped RNA virus of the Hepevirus genus. The four major genotypes (GI to GIV), all belonging to a single serotype, are known to infect humans. While GI and GII are restricted to humans, GIII and GIV are zoonotic and may infect animals (swine, chickens, deer, mongooses, and rabbits), as well as humans, in both industrialized and non-industrialized countries [96]. Symptoms include malaise, anorexia, abdominal pain, arthralgia, fever and jaundice. The

incubation period for HEV varies from 14 to 63 days. HEV infection usually resolves in 1-6 weeks after onset. The fatality rate is 0.5-3%, except in pregnant women, for whom the fatality rate can approach 20-25%. HEV has been included in the National Institute of Allergy and Infectious Diseases NIAID List of Emerging and Re-emerging Diseases (Group I-Pathogens newly recognized in the past two decades). Illnesses associated with HEV are rare in industrialized countries, with most infections being linked to international travel. HEV is transmitted via the fecal-oral route and is easily spread by water contaminated with human fecal matter. Large waterborne outbreaks with high attack rates among young adults have been described in regions characterized by poor sanitary conditions. Since the first retrospectively documented hepatitis E outbreak in India in 1955-1956, many large waterborne outbreaks have been reported in Asia and Africa [97], affecting up to 79 000 and even 119 000 persons in India and China in 1991, respectively. Most of the outbreaks occurred after monsoon rains, heavy flooding, contamination of well water, or massive uptake of untreated sewage into city water treatment plants. No reports of HEV linked to recreational water were found in the published literature. Two cases of HEV were reported in persons who swam in the River Ganges, but they also drank unboiled or unfiltered water while in India [11]. In industrialized countries, while there have been sporadic cases of locally acquired hepatitis E, no epidemics have been reported. HEV has been detected, however, in different water environments, including urban sewages, in Spain, Italy, France and the United States [98-102]. Moreover, infectious HEV particles have been reported to occur in sewage, indicating the existence of a potential public health risk from the contamination of surface water with HEV [103]. HEV has also been detected in rivers [104, 105] and in bivalves [106].

Influenza virus

Influenza is a highly contagious, acute respiratory illness caused by a virus, member of the *Orthomyxoviridae* family, containing a segmented negative-sense RNA genome. The segmented nature of the genome allows for the reassortment or exchange of segments between two virus strains co-infecting the same cell; thus, influenza viruses are constantly reemerging through changes in their genome. All subtypes of influenza A viruses (H1 to H16 and N1 to N9) have been isolated from wild waterfowl. Most infected birds are asymptomatic, even when they are excreting large quantities of infectious virus in their feces as well as in their saliva and nasal secretions, and act as "silent" reservoirs of the virus. Domestic waterfowl (*e.g.*, ducks) may also act as a two-way intermediary in the transmission pathway of avian influenza between wild waterfowl and domestic terrestrial poultry (*e.g.*, chickens) [107]. Viral transmission occurs mainly by direct contact between infected birds and essentially the respiratory tract of susceptible hosts, but the role of an indirect waterborne transmission linked to feces-contaminated water has also been confirmed [108, 109]. It had been thought that avian viruses, although highly pathogenic for domestic poultry, did not replicate efficiently or cause disease in humans. However, human cases of avian influenza have recently become increasingly frequent. The threat posed by highly pathogenic avian influenza A H5N1 viruses to humans remains significant, given the continued occurrence of sporadic human cases, the endemicity in poultry populations in several countries, and reassortment, which may produce novel viruses of potential threat to public health. Contaminated lakes and ponds play an essential role as environmental virus reservoirs. Avian influenza virus has been isolated from lakes and pond waters and other water environments, where they can persist for extended periods of time [107-111]. There is very little information on the role of water in the transmission of influenza viruses among waterfowl or to other animals, including humans. De Jong described two simultaneous H5N1 cases in a single family in Vietnam, suggesting that exposure to possibly contaminated canal water via swimming or washing may have resulted in infection. The role of water in transmission however, could not be confirmed [112]. More recently, two studies identified contaminated water as a potential risk factor for H5N1 infection in a Cambodian village [111, 113]. Nevertheless, observational and analytical studies have not yet identified exposure to a contaminated water environment as an established risk factor for influenza H5N1.

Coronavirus

Coronaviruses, of the *Coronaviridae* family, are enveloped, single-strand RNA viruses that range from 60 to 220 nm in size. They are primarily respiratory pathogens, a frequent cause of the common cold in both children and adults. Although its major source of transmission is person-to-person contact through respiratory secretions, the fecal-oral transmission may be possible as well. Coronaviruses were not considered of concern for waterborne transmission, until a new coronavirus, the causative agent of severe acute respiratory syndrome (SARS), was detected in the feces of infected patients. In addition, large clusters of cases suggest the possibility of environmental contamination via sewage or ventilation systems [114]. In 2003, during the investigation of an outbreak in Hong Kong, transmission by aerosolized wastewater was suspected [115]. A recent study investigated the survival of representative coronaviruses in tap water and wastewater [116]. Inactivation of coronaviruses in the test water was highly dependent on temperature, level of organic matter, and presence of bacteria. Coronaviruses were inactivated faster in water at 23 °C (10 days) than in water at 4 °C (> 100 days); they died off rapidly in wastewaters (2-4 days). Research is still needed to shed light on the persistence of this virus in the environment, its zoonotic reservoirs, and its potential transmission through waterborne routes.

Polyomaviruses

Human polyomaviruses (HPyV) are members of the *Polyomaviridae* family. Polyomaviruses are approximately 38 to 43 nm in size. Their double-stranded DNA is thought to contribute to their heat stabil-

ity and increased resistance to UV light treatment. Polyomavirus infections appear to be lifelong, but are generally asymptomatic in healthy individuals. In the immunocompromised host, however, reactivation of the latent viruses appears to occur in the kidneys and brain tissue leading to potential tumor development. Five HPyV have been known for some time (*i.e.*, JCV, BKV, MCV, WUV, and KIV) and four additional types have been recently characterised [117]. Polyomaviruses of primary concern include the JC virus (JCV), BK virus (BKV) and the Merkel cell virus (MCV), responsible for diseases such as progressive and fatal multifocal leukoencephalopathy (brain cancer), nephropathy (kidney cancer), and Merkel cell carcinoma (MCC), a rare but aggressive skin cancer, respectively. BK virus and JC virus are ubiquitous in the human population with up to 90% and 60%, respectively, of the adults having antibodies against these viruses [117]. MCC is extremely unusual before the age of 50 and people at risk include those with fair skin, excessive UV light exposure, and immunosuppressed patients. The incidence rate is 0.44 cases per 100 000 individuals in the USA and 0.15 cases per 100 000 individuals in Japan; the number of MCC cases has, however, tripled the last 15 years [118]. Exposure to HPyV occurs early in childhood, generally by the age of five to 10. Polyomaviruses are excreted in the urine and feces of infected individuals, both healthy and symptomatic, and are thus subject to environmental transmission via the waterborne route. JC and BK PyVs have been detected in urban sewage from various geographical areas [119, 120]. Recently Bofill-Mass *et al.*, [121] analyzed the presence and characteristics of newly described human HPyVs (KI, WU and Merkel cell PyV) in urban sewages and river waters. This study indicates that the MCV, a virus strongly associated with human cancer, is prevalent in the population and may be disseminated through the fecal/urine contamination of water.

Picobirnavirus

Human picobirnavirus (HPBV) are fairly small (35 nm), non-enveloped, spherical viruses. They have been found in a wide range of hosts, including humans, with or without GE. These viruses have not been successfully cultured in the laboratory, and their pathogenesis is unknown. High levels of occurrence in wastewater samples have been reported. Symonds and coworkers detected HPBV in 100% of raw sewage samples and 33% of final effluent samples [122]. Whether the presence of HPBV in aquatic environments is specific to human fecal contamination or whether animals may act as a source of contamination is unclear. The authors suggested that these viruses could be used, along with AdVs as potential markers of fecal contamination. However, a recent study detected HPBVs in only 25% of wastewater samples, casting doubt on their suitability as indicators of fecal contamination in water [123]. Further research will be needed to determine if these candidate viruses have the necessary characteristics of a microbial water quality indicator.

Papillomaviruses

Papillomaviruses are small epitheliotropic viruses detected in all vertebrates. Human Papillomaviruses (HPVs) belong to the *Papillomaviridae* family and are distributed over five genera (alpha, beta, gamma, mu and nu). They are small (approx. 50-60 nm), with a circular double stranded DNA genome measuring 7-8 kb in length. Infections due to papillomaviruses are common and lead to a wide variety of clinical manifestations that involve the epidermal surfaces, including common warts, palmoplantar warts, oral warts and genital warts. Strong evidence indicates that certain papillomaviruses are involved in cervical and genital cancers. Some have also been implicated in laryngeal/oral cancer and some lung cancers [124]. Recent evidence suggests that epitheliotropic viruses can find their way into sewage as enteric viruses [125]. A recent study detected HPVs in the vast majority (81%) of sewage samples, with a wide range of genotypes belonging to both the alpha and the beta genus, including putative novel genotypes [126]. Moreover, not only cutaneous HPVs, but also three of the most important anogenital types, such as the oncogenic HPV-16 and the low-risk HPV-6 and HPV-11 genotypes, were detected [126]. Although large-scale studies will be required before the health significance of the presence of HPVs in the environment is fully understood, these data pave the way for investigations on HPV transmission through contaminated water. In fact, this could reveal a hitherto unexplored mode of HPV transmission which may account for anogential HPV infection among people who had never been sexually active (*e.g.*, children and virgins).

CONCLUSIONS

Globalization, new technologies, and the genetic evolution of pathogens, humans, and vectors stimulate the emergence of new microbial threats to water quality. Despite the progress in water and wastewater treatment technology, waterborne diseases continue to have far reaching public health consequences in both non-industrialized and industrialized countries. Efforts to improve the detection, investigation, and reporting of outbreaks at the local and national levels worldwide, modeled on the WBDOSS surveillance system, are crucial to identify the causes of outbreaks and to understand the environmental factors contributing to these outbreaks.

Future studies are needed to provide valid and reproducible methods for the detection of waterborne viral pathogens in order to determine the extent of contamination of water environments, the types of pathogens involved and the correlation between viral contamination and environmental factors. Also, quantitative microbial risk assessment (QMRA) analysis should be improved, using water quality data, pathogen-specific characteristics, prevalence data, and exposure data. Such information will lead to a better understanding of the health risks related to water systems, and to improved methods of control.

In summary, research efforts to mitigate the effects of infectious threats, focusing on improved surveillance

and diagnostic capabilities, including reliable viral indicators of fecal contamination of water, are crucial.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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Sanitary problems related to the presence of *Ostreopsis* spp. in the Mediterranean Sea: a multidisciplinary scientific approach

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Abstract. The increased presence of potentially toxic microalgae in the Mediterranean area is a matter of great concern. Since the end of the last century, microalgae of the genus *Ostreopsis* have been detected more and more frequently in the Italian coastal waters. The presence of *Ostreopsis* spp. has been accompanied by the presence of previously undetected marine biotoxins (palytoxins) into the ecosystem with the increased possibility of human exposure. In response to the urgent need for toxicity characterization of palytoxin and its congeners, an integrated study encompassing both *in vitro* and *in vivo* methods was performed.

Key words: Ostreopsis, palytoxin (PLTX), acute toxicity, myotoxicity, cutaneous toxicity.

Riassunto *(Problemi sanitari relativi alla presenza di* Ostreopsis *spp. nel Mar Mediterraneo: un approccio scientifico multidisciplinare)*. La sempre maggiore presenza di microalghe potenzialmente tossiche nell'area mediterranea è motivo di grande preoccupazione. Dalla fine del secolo scorso, microalghe appartenenti al genere *Ostreopsis* sono state isolate con sempre maggiore frequenza nelle acque costiere italiane. La presenza di specie di *Ostreopsis* è stata accompagnata dalla comparsa di biotossine marine (palitossine), mai isolate prima nell'ecosistema, con conseguente aumento della probabilità di esposizione umana. In risposta al bisogno urgente di caratterizzazione della tossicità della palitossina e dei composti strutturalmente correlati, è stato scelto un approccio di studio integrato tra metodiche *in vitro* e *in vivo*.

Parole chiave: Ostreopsis, palitossina (PLTX), tossicità acuta, miotossicità, tossicità cutanea.

INTRODUCTION

Microalgae of *Ostreopsis* spp. are unicellular epiphytic benthic dinoflagellates [1, 2]: originally, they were thought to colonize only tropical and sub-tropical areas, but they are now being detected more and more frequently in temperate seas [3, 4], suggesting their geographic spread in the benthic environment. Concern about the distribution of *Ostreopsis* is motivated by its high potential for toxicity [4]. The entrance of potentially toxic dinoflagellates into the ecosystem can have impacts at several levels. In general, sanitary and economic consequences, often tightly connected, are of the greatest concern. In the Mediterranean region, appearance and proliferation of *Ostreopsis* spp. were first recorded in the late 1970s and 1980s [5, 6]. In Italy, the presence of *Ostreopsis ovata* was recorded for the first time in 1994, along the coasts of the Lazio region [7]. Since then, the presence of *Ostreopsis* spp., most often *O.* cf. *ovata*, has been recorded several times along the Italian coastline [8-21], including the northern areas, such as the Gulf of Genoa [19, 22, 23] and the Gulf of Trieste *(Figure 1)* [19, 24, 25].

Globally, attention shifted toward *Ostreopsis* in 1995, when Dr. Takeshi Yasumoto's group isolated and characterized PLTX-like compounds from *O. siamensis* [26-27]. Until this time, the origin of palytoxins was thought to be soft corals of the genus Palythoa [28]. Since then, there has been increasing consensus in the scientific community regarding microalgae as at least one producer of the toxins, even though the biosynthetic pathways are still unclear and represent a field of open and ongoing research. In addition to *O. siamensis*, putative PLTX and analogues have been isolated from O. mascarenensis [29] and *O. ovata* [30-35]. Extensive studies on *Ostreopsis* samples has led to the identification of several PLTX congeners *(Figure 2)*, including ostreocin-D [26, 27], mascarenotoxins [29-34] and several ovatoxins, denoted ovatoxin-a, -b, -c, -d, -e [30-34] and ovatoxin-f [35].

Palytoxin is considered among the most toxic compounds of natural origin ever isolated. It impairs the function of the Na^+/K^+-ATPase [36-39], whose physiological activity is of crucial importance for eukaryotic cells. Since 2006, our research group at

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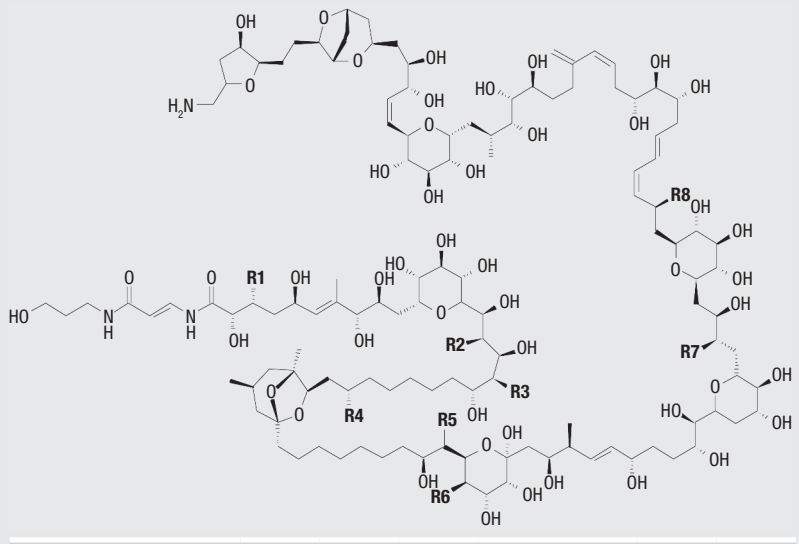
Fig. 1 | *Schematic representation of the sites of* Ostreopsis *blooms along the Italian coasts. (Data sources: [7-21]; http://arpa.sicilia.it and A. Penna, personal communication).*

the University of Trieste has followed the PLTX-phenomenon and added the study of PLTXs to the ongoing research on other algal toxins. This mini-review is mainly focused on the results obtained by our research group on this topic. Considering the potential different route of exposure to these toxins and the possible scenarios of intoxication, starting from the available data, an integrated *in vivo* and *in vitro* approach, including toxicological, physiological, cellular biology and biochemical studies, was adopted. This mini-review mainly summarizes the results of these studies, aimed to characterize PLTXs toxicity, and to identify the target organs and the mechanism at the basis of the toxic effects, useful also to provide a suitable therapeutic approach.

Potential exposure to Ostreopsis *spp. and the related toxins into marine aerosols and seawater: possible health effects*

In the Mediterranean area, the increasing proliferation of *Ostreopsis* spp. along the coastlines was accompanied by the occurrence of human intoxication [10, 40-43]. In particular, human exposure to marine aerosol and/or seawater concomitantly to *Ostreopsis* proliferations was associated with an illness in which symptoms involved mainly the upper respiratory tract [40]. The cause-effect correlation between the cases of malaise and the involvement of algal toxins has not been completely clarified: in fact palytoxins were never detected in marine aerosol so far, even though these toxins were quantified in field algal samples [31]. Furthermore, although *Ostreopsis* cells concentrations were determined in seawater, these data are not predictive for human risk since dinoflagellates do not always produce the same amount of toxins, if any [25]. *Ostreopsis* cell debris can be also present in the marine aerosol and their contribution to the ef-



Toxin	R1	R2	R3	R4	R5	R6	R7	R8
PLTX	CH_3	OH	OH	CH_3	H	OH	OH	OH
OST-D	H	OH	H	H	OH	H	OH	OH
OVA-a	CH_3	H	OH	CH_3	OH	H	H	OH
42-OH-PLTX	CH_3	OH	OH	CH_3	OH	OH	OH	OH

Fig. 2 | *Structure of palytoxin (PLTX), ostreocin-D (OST-D), ovatoxin-a (OVA-a) and 42-hydroxy-palytoxin (42-OH-PLTX).*

fects on human health cannot be excluded. Anyway, the recurrence of sanitary problems associated with *Ostreopsis* blooms suggests a relationship between these phenomena [43].

Along the Italian coastlines, the first documented health problems associated with *Ostreopsis* blooms were described as general malaise in people exposed both to seawater and/or marine aerosol in Tuscany [9] and Apulia [8]. Later, symptoms such as rhinorrhea, cough, dyspnea and fever, associated with blooms along the Bari coast, were described in more detail by Gallitelli, *et al.* [10]. Similar symptoms were observed along the Spanish and French Mediterranean coasts, accompanied by ocular irritation, headache and, in some cases, by fever [41, 42]. Other anecdotal descriptions of respiratory problems following marine aerosol exposure during *Ostreopsis* blooms have also been reported along the Mediterranean coast [25, 43, 44]. The most serious sanitary problems occurred on the Liguria coast in summer of 2005 [22, 40] and repeated, with a lower intensity, in 2006 [40]. In July 2005, more than 200 people enjoying the Genoa beach and promenade suffered an unusual influenza-like syndrome, characterized by a wide spectrum of symptoms such as fever, sore throat, cough, dyspnea, headache, nausea, rhinorrhea, lacrimation, vomiting and dermatitis. Approximately 20% of patients required hospitalization (1-3 days), and some of them needed the intensive care unit of the local hospitals [40]. This occurrence represents the most severe incident described to date in terms of both the number of people affected and for the severity of the symptoms.

In addition to the problems at the respiratory level, skin irritation was frequently observed after aerosol exposure and/or seawater contact during *Ostreopsis* blooms. Indeed, in summer 2005, concomitant with marine aerosol exposure during *Ostreopsis ovata* blooms in Genoa (Northern Italy), the incidence of dermatitis was 5% [40]. Erythematous dermatitis was also observed by Gallitelli (personal communication) in patients exposed to marine aerosols during *Ostreopsis* blooms, along Apulia coasts (Southern Italy) [43].

Although the actual cause of this dermatitis has not yet been unequivocally determined, dermal toxicity has been associated to PLTX-like molecules contaminating other marine organisms [43, 45]. For instance, skin toxicity has been reported after handling zoanthids *(Palythoa)* used as aquarium decorative corals: persistent signs of dermotoxicity and perioral paresthesia were attributed to PLTX in a patient with intact skin [46]. Another case of skin toxicity due to handling PLTX-containing zoanthid corals (*Parazoanthus* spp.) involved a patient who cut his fingers while cleaning his aquarium. Dermal distress with swelling, paresthesia and numbness around the site of injury, as well as systemic symptoms, were recorded [47]. Despite the reports on human dermal toxicity attributed to PLTXs, very little data regarding skin toxicity are presently available in the scientific literature. Our group contributed to the elucidation of the cutaneous effects of the parent compound PLTX: an *in vitro* toxicity study was carried out using the human HaCaT keratinocytes [48], a predictive model for the evaluation of skin toxicity and an ideal model for first-round screening of dermotoxic agents [49]. The cytotoxicity of PLTX on HaCaT cells was investigated after a short time exposure (4 h) and different cellular endpoints were evaluated. PLTX reduced mitochondrial activity (MTT assay), cell viability (sulforhodamine B assay) and plasma membrane integrity (LDH leakage), albeit with different potencies (EC_{50} = 6.1 ± 1.3 x 10^{-11} M, 4.7 ± 0.9 x 10^{-10} M and 1.8 ± 0.1 x 10^{-8} M, respectively). These data suggest that the sequence of intracellular events following the interaction of PLTX with its molecular target includes mitochondrial damage, causing a reduction in cell viability and plasma membrane rupture, with resulting leakage of LDH. Moreover, mitochondrial dysfunction was tentatively associated with oxidative stress, since PLTX induced superoxide anion accumulation after only 1 h exposure [48]. All these effects were inhibited by the presence of ouabain, a cardiac glycoside that inhibits PLTX binding to its molecular target (Na^+/K^+-ATPase). These data demonstrated the dependency of PLTX cytotoxicity on the interaction with the pump, which is transformed by PLTX into a non-selective cationic pore [37-39, 50]. The main consequence of this interaction seems to be a sustained intracellular overload of Na^+, followed by an overload of Ca^{2+} [51-54]. Consequently, the mechanism of PLTX cytotoxicity was investigated, with particular attention to the ionic imbalance induced by the toxin. On HaCaT cells, removal of Na^+ from the cell medium almost completely abolished: *i)* PLTX-induced oxidative stress, *ii)* impairment of mitochondrial activity and *iii)* appearance of morphological changes, demonstrating that intracellular Na^+ accumulation is the first and crucial step in mediating PLTX-induced early cell damage. By contrast, Ca^{2+} withdrawal did not affect PLTX-induced oxidative stress or cell morphology, confirming the Na^+-dependency of these effects on HaCaT keratinocytes [48].

Potential exposure to Ostreopsis *spp. and related toxins via seafood: possible health effects*

The presence of toxic *Ostreopsis* species into the ecosystem is related to the entrance of their toxins into the food web. The accumulation of biotoxins in the food web is a common, naturally occurring phenomenon [55], but it may lead to significant concentrations of toxic compounds in edible organisms and represent a potential threat to human health. Palytoxins are no exception and have been detected in several species of crustaceans, fish, mollusks and echinoderms, even if the consequences of their accumulation seem to differ from area to area [56-65]. In tropical and subtropical areas, accumulation of PLTXs in fish and crustacean lead to some cases of intoxication, even with lethal outcomes [43, 57-60]. In Madagascar, for instance, a lethal human intoxication

has been reported resulting from ingestion of PLTX-contaminated sardine sharing habitat with *Ostreopsis* [59]. In the Mediterranean area, contamination involved mainly mollusks and echinoderms, such as shellfish and sea urchins [19, 61-66] but, to date, no case of human intoxication has been described. In Italy, palytoxins were detected mainly in the frame of monitoring programs [19, 64]. The presence of ovatoxin-a (303-625 μg/kg) has been reported mainly in wild mussels collected along the rocky Italian coasts [64]. Similarly, in France, contamination reached 450 μg PLTX equivalents/kg of total flesh of sea urchins and 230 μg PLTX equivalents/kg of total flesh of mussels [65]. PLTXs and related toxins are not routinely tested, because no regulation at Italian or European level currently include them in the monitoring programs. Moreover, in Mediterranean Sea, the most abundant PLTXs detected in seafood are ovatoxins, and in particular ovatoxin-a, not yet available in sufficient amounts for oral toxicological studies, that are necessary for regulatory purposes.

Anyway, EFSA suggested 30 μg PLTXs (sum of PLTX and ostreocin-D)/kg shellfish meat as maximum level in shellfish [63]. However, this value is based on the limited available toxicological studies carried out in mice with the pure toxins after oral, sublingual and intratracheal exposure, the last one being not representative in humans for this evaluation. Other toxicological studies on PLTX were initially performed immediately after its discovery: no effects were observed in rats after oral administration of 40 μg/kg of a compound, which molecular weight was 3300 Da [67], nearly 500 Da higher than that currently reported for PLTX. More than thirty years later, an LD_{50} = 510 μg/kg was estimated for PLTX in mice, evaluating only lethality as endpoint of toxicity [68]. Subsequently, Ito and Yasumoto [69] reported tissue damages induced by the oral administration of PLTX or ostreocin-D (200 or 500 μg/kg). To implement the toxicological characterization, the acute oral toxicity of PLTXs in mice was evaluated increasing the dosages and expanding the panel of endpoints (*i.e.* histological and hematoclinical analyses). The toxicity was initially evaluated, within 24 h after the administration, on the parent compound PLTX and it was found to be strictly dose-related [70]. Later, the study was repeated on 42-hydroxy-PLTX [71], chemically characterized in 2009 [72] *(Figure 2)*. Similar in structure, PLTX and 42-hydroxy-PLTX also resulted in similar toxicity and symptoms. During the observation period, some of the mice presented scratching, jumping, paralysis of the hind limbs, respiratory distress, occasionally accompanied by cyanosis and died within 24 h from the administration of the toxins. Histological analysis revealed decreased glycogen content in hepatocytes. Mice that survived the treatment exhibited several degrees of inflammation of the mucosa in the forestomach [70, 71].

In animals treated from the dose of 600 μg/kg, hematochemical analysis revealed alteration in plasma levels of creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and aspartate transaminase (AST), suggesting involvement of the muscular tissue in the toxicity of PLTX and 42-hydroxy-PLTX [70, 71]. In animals treated with PLTX, dose-dependent ultrastructural alterations of skeletal and cardiac muscle were also observed. The identification of skeletal muscle as one of the targets for PLTX was in agreement with the epidemiological data [43], which revealed that, in the majority of human cases, muscular problems and myalgia were reported as distinctive features [57, 58, 60]. For this reason, cultures of mouse skeletal muscle cells were chosen as a suitable model for the deeper investigation of the mechanism of action of both PLTX [73] and 42-hydroxy-PLTX (Del Favero *et al.*, in preparation). As mentioned above, PLTX is known to impair the activity of the Na^+/K^+ pump, with dramatic consequences on cellular ionic homeostasis [36-39, 50]. As well as high toxicity, quite common to all the cells models tested so far [74], PLTXs triggered an uncontrolled intracellular calcium ($[Ca^{2+}]_i$) increase [72, 73] and morphological alterations [73]. These events seemed to be strictly related to the development of the toxic insult [73]. The $[Ca^{2+}]_i$ increase consisted of a transitory Ca^{2+} response (transient phase) followed by a slower and more sustained $[Ca^{2+}]_i$ increase (long-lasting phase). The transient phase was sustained by the *i)* activation of voltage-dependent Ca^{2+} channels, *ii)* Na^+/Ca^{2+} exchanger (reverse mode) and *iii)* Ca^{2+} release from intracellular stores with no influence on the PLTX-mediated toxicity. The long-lasting phase seemed to be sustained by the activation of stretch-activated channels and represents a crucial step in the development of the myotoxic insult [73]. PLTXs did not only severely impair cellular viability, but also altered the functional properties of skeletal muscle cells, such as the ability to respond to physiological stimuli [73] (Del Favero *et al.*, in preparation). On the whole, the skeletal muscle cell cultures allowed the characterization of the ionic disequilibrium triggered by PLTXs, opening new insight into the mechanism of action of PLTX at the single cell level.

The alterations at the muscular level observed in mice after acute PLTX oral exposure, together with the epidemiological observations in humans (lethality, muscle cramps, myalgia, and cardiac alterations) suggest PLTX absorption in the gastrointestinal tract after oral exposure. Thus, information on the gastrointestinal absorption of PLTX seemed to be pivotal for a rational risk assessment. Since no toxicokinetic data on PLTX were available, an *in vitro* study was carried out for the evaluation of PLTX absorption through the intestinal barrier: to this aim the human Caco-2 cell line was used. However, Caco-2 cells are one of the most sensitive cell models for PLTX, presenting reduced viability at the sub pico-molar range (EC_{50} = 8.9 ± 3.7 x 10^{-12} M after 4 h exposure, MTT assay). Unfortunately, the high sensitivity of this model precluded the possibility of evaluating PLTX absorption [75].

DISCUSSION AND CONCLUSIONS

The relative rapidity that characterizes the entrance of new species of potentially harmful microalgae in the Mediterranean ecosystems represents an immense challenge from the scientific and regulatory point of view. The data necessary for the evaluation of real toxicological hazard beneath naturally occurring phenomena, such as algal blooms, require time and resources. Thus, even though *Ostreopsis* appeared in Mediterranean waters over 30 years ago [5], the toxicological consequences of exposure to its suite of toxins is still an open research field.

A multidisciplinary scientific approach for the toxicological characterization of PLTXs based on literature and epidemiological data was followed. Initially, wider *in vivo* acute toxicity studies allowed to individuate the skeletal muscle as one of the main targets of PLTXs toxicity, in agreement with human symptoms. Although no structural alterations were observed in mice, the sharp increase in CPK, K^+ and LDH plasma levels suggested the skeletal muscle involvement, subsequently confirmed by ultra-structural changes. Further *in vitro* studies on skeletal muscle cells contributed to the elucidation of PLTX effects at functional level and to the characterization of its mechanism of action, opening new perspectives.

The lack of toxicokinetic data on PLTX and the difficulty of predicting absorption and distribution in the body is still a challenge for the comprehension of the hazard associated with PLTXs in the food web. Moreover, the accumulation of the toxins in several edible marine species [19, 56-65] opens the possibility of repeated human exposure through contaminated seafood collected in the same area.

Considering exposure routes different from the oral one, the cutaneous toxicity was characterized using an *in vitro* approach. The high toxicity of PLTX on skin keratinocytes [48] raises valid concerns about the potential human exposure to PLTX-related toxins in seawater and needs to be further investigated. These data will help the prevention of toxicity for people exposed to seawater during *Ostreopsis* blooms, either professionally or recreationally. Toxicological evaluation of PLTX-like compounds after inhalational exposure remains one of the most crucial issues, and should be addressed as soon as possible by the scientific community.

In conclusion, sanitary problems related to *Ostreopsis* spp. could be due to the entrance of previously absent toxins into the food web and to human exposure to marine aerosols and/or seawater during large algal blooms. A multidisciplinary approach to the problem should be further adopted for their complete exploitation and evaluation. The number of known toxins is constantly increasing, requiring additional efforts for toxicity characterization. Studies are urgently needed to evaluate the effects of these toxins after repeated oral exposure. Another crucial point is the characterization of the oral toxicity of the new ovatoxin analogues as well as of their inhalational toxicity.

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Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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Emerging health issues of cyanobacterial blooms

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Abstract. This paper describes emerging issue related to cyanobacterial dynamics and toxicity and human health risks. Data show an increasing cyanobacteria expansion and dominance in many environments. However there are still few information on the toxic species fitness, or on the effects of specific drivers on toxin production. Open research fields are related to new exposure scenario (cyanotoxins in water used for haemodialysis and in food supplements); to new patterns of co-exposure between cyanotoxins and algal toxins and/or anthropogenic chemicals; to dynamics affecting toxicity and production of different cyanotoxin variants under environmental stress; to the accumulation of cyanotoxins in the food web. In addition, many data gaps exist in the characterization of the toxicological profiles, especially about long term effects.

Key words: toxic cyanobacteria, human health, climate change, toxins, risk assessment.

Riassunto *(Problemi sanitari emergenti legati a fioriture di cianobatteri)*. Questo articolo esamina problemi emergenti relativi alla dinamica e alla tossicità di cianobatteri in relazione ai rischi per la salute umana. È nota la crescente diffusione dei cianobatteri in molti ambienti. Tuttavia, ci sono tuttora poche informazioni sulle specie tossiche e sui fattori in grado di modulare la tossicità. Sono aree di ricerca emergenti: nuovi scenari di esposizione (cianotossine nelle acque usate per emodialisi e negli integratori alimentari); nuovi modelli di co-esposizione a cianotossine e tossine algali e/o prodotti chimici artificiali; dinamiche di tossicità e produzione delle diverse varianti di cianotossine in condizioni di stress; trasferimento di cianotossine nella catena alimentare. Inoltre, sono ancora presenti lacune nella caratterizzazione dei profili tossicologici di molte tossine, specialmente sugli effetti a lungo termine.

Parole chiave: cianobatteri tossici, salute umana, cambiamenti climatici, tossine, valutazione del rischio.

INTRODUCTION

Cyanobacteria are a morphologically diverse group of photosynthetic prokaryotes that occupy a wide range of niches, from freshwater to hydrothermal vents, from desert rocks to Antarctic lakes *(Figure 1)*. They have been reported in freshwater lakes, basins, rivers, irrigation channels, brackish and sea waters, salty lakes, as pelagic or benthic organisms. Several cyanobacteria species produce toxins as secondary metabolites, which can impact on ecosystems, animal and human health [1, 2]. On the basis of toxin production, to which human can be exposed via different routes, WHO has listed cyanobacteria among the emerging health issues, although not considering them as emerging pathogens, since their possibility to infect human beings has never been reported so far.

Cyanobacteria are expanding geographically and now threaten the ecological integrity and sustainability of some of the world's largest and most resourceful water bodies, including Lakes Victoria (Africa), Erie (US-Canada), Okeechobee (Florida, USA), Taihu (China), Kasumigaura (Japan), the Baltic Sea in Northern Europe and the Caspian Sea in West Asia [3]. The present and possible future cyanobacterial bloom expansion can be attributed to nutrient over enrichment in watersheds with relevant human activities [3] and changing climatic conditions [4].

Predicting future scenarios is a major challenge to ensuring protection of human health. It is necessary to define the potential risk associated to cyanobacterial presence in different environment in relation to exposure routes. As a consequence, it will be possible to identify the appropriate management strategies to be adopted to protect water resources, mitigating the negative ecological and biogeochemical impacts and economic losses both in the short and long run. It has been estimated that only in the United Sates cyanobacterial blooms result in losses of recreational, drinking and agricultural water resources that are worth > $ 2 billion annually [5].

A lot of studies have been published on the occurrence of cyanobacteria and their ability to produce cyanotoxins in surface waters; they have been extensively reviewed in dedicated publications on both ecological and toxicological aspects [1, 2, 6, 7].

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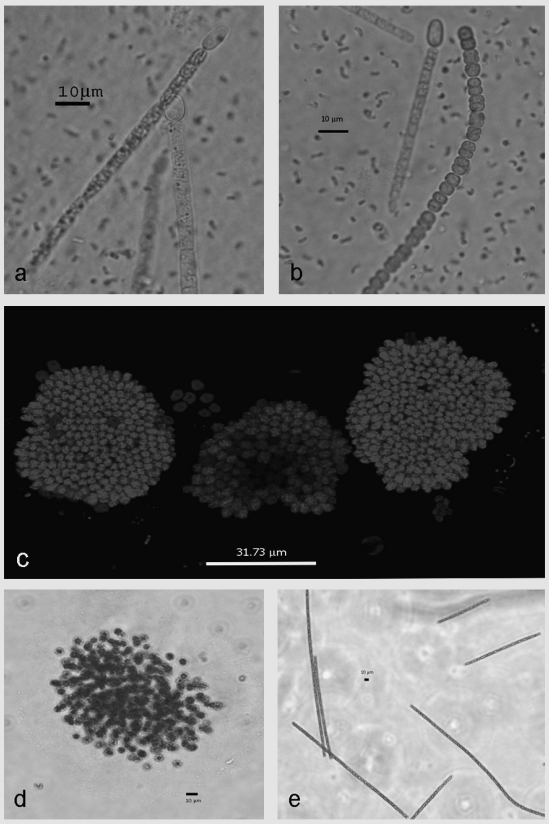

Fig. 1 | *Some examples of different morphologies. a)* Cylindrospermopsis raciborskii *filaments; the Australian strain and the strain from North America produce cylindrospermopsins and saxitoxins, respectively; b)* Nostoc *sp. filaments, microcystins producer; c)* Woronichinia naegeliana*, unicellular colonies, microcystins producer; d)* Microcystis botrys *unicellular colonies, microcystins producer; e)* Planktothrix rubescens *filaments, microcystins producer (Photos by Stefanelli Mara).*

The number of papers on the issue has dramatically raised up in the last few years, after the increased occurrence of cyanobacterial blooms *(Figure 2)* and the awareness of their possible health impact, but also due to the continuously reported new findings. Indeed, the list of cyanobacterial species/strains appears to be far to come to an end, and at present it is not clear which is the proportion of known cyanotoxins vs unknown ones. A number of data gaps can be identified in the toxicological profile of the different cyanotoxins known so far, as well as in the description of exposure scenarios for humans.

Although many data are available on cyanobacterial ecology, the most studied aspect so far, from the health perspective there are still many issues that have not been clarified and, most importantly, they can vary depending on the species and on the environmental area of occurrence. The knowledge of these aspects should be the solid scientific ground in developing models to better predict the occurrence of blooms and their toxicity, that would limit the monitoring activities saving human and economic resources, and consequently to identify efficient prevention measures.

This paper briefly summarizes the current knowledge on toxic cyanobacteria with specific focus on emerging issues and main gaps in the area of risk assessment. After the description of main habitats affected by toxic cyanobacteria bloom, other than the very well known freshwater rivers and lakes, exposure scenarios and effects on human health will be addressed. The impacts of climate changes on cyanobacteria diffusion and toxicity, and hence on human exposure, are addressed in Funari *et al.* [8], in this same journal issue.

DISTRIBUTION OF CYANOBACTERIA AND THEIR TOXICITY

Respect to the past, data are available indicating a wider distribution and increasing dominance of cyanobacteria in many environments. However there are much less clear indications on the fitness or diffusion of the toxic species, or on the effects of specific drivers on toxin production.

Cyanobacteria can bloom not only in euthrophic habitats, but also in oligotrophic environment thanks to various strategies. Some cyanobacteria, like *Anabaena*, and other filamentous ones, can resist in nitrogen (N) depleted waters because are diazotrophic, that is they can fix atmospheric nitrogen. For these organisms, phosphorous (P) seems the key nutrient for bloom formation, but they can still outcompete other organisms in environments with low dissolved P thanks to the formation of akinetes, sedimented in the bed of the water basin, which allow the organisms to get P from sediments

Fig. 2 | *Two blooms of* Microcystis aeruginosa *in temperate lakes.*

[9]. Otherwise, *Anabaena* and *Cylindrospermopsis raciborskii* can also use organic form of P [9]. *Microcystis aeruginosa* has a high affinity for dissolved inorganic phosphorous (DIP), which allows it to bloom in low DIP environment [9].

Other than high affinity nutrients systems and/or their own ectoenzymatic activity, cyanobacteria can utilize the nutrients mineralized by ectoenzymatic activity of other organisms. It has been demonstrated that cylindrospermopsin (CYN) produced by *Aphanizomenon ovalisporum* induces the release of alkaline phosphatase in other phytoplankton species. Under phosphorus limitation the production of CYN, coupled to a high affinity P uptake system, enables *Aphanizomenon* to take advantage of the enzymatically released P [10]. In a small oligotrophic lake in Central Italy, with undetectable inorganic and significant concentration of organic nitrogen, *Planktothrix rubescens* overcame nitrogen limitation thanks to bacterial amminopeptidase and chitinase activity [11], releasing small oligopeptides and organic compounds in the surrounding water. Therefore the strategic role of phytoplankton bacteria should be carefully considered, especially for oligotrophic water bodies not to underestimate the possible occurrence of cyanobacterial blooming episodes.

Marine planktonic cyanobacteria

Cyanobacteria occupy a wide range of niches in marine ecosystems in tropical and temperate regions, where they occur along eutrophic maritime coasts as well as in the oligotrophic open ocean representing more than 50% of phytoplankton biomass and carbon production [12]. According to Fristachi and Sinclair [13] the knowledge of adverse effects of cyanobacteria in the marine environments has only recently begun to be recognized. It is possible that in the future, as more information will be available, we will realize that the frequency of blooming and the impact for both human health and the environment are much higher.

Marine planktonic cyanobacteria are classified into three morphological and functional groups: unicellular (*Synechococcus, Prochlorococcus, Synechocystis, Aphanothece* and *Merismopedia*), non-heterocystous filamentous (*Lyngbya, Oscillatoria, Phormidium, Spirulina* and *Trichodesmium*), some of which can fix nitrogen, and heterocystous filamentous (*Anabaena, Aphanizomenon, Nodularia* and *Richelia*), all N_2-fixing organisms.

Among planktonic cyanobacteria, *Trichodesmium* is the most abundant and well studied diazotrophic cyanobacteria in the open ocean and in coastal water as well. It forms extensive blooms in oligotrophic, tropical, and subtropical oceans [14], usually characterized by a very stable water column and a deep upper mixed layer (about 100 m). The success of *Trichodesmium* in these low-nutrient water includes its capability to fix nitrogen, its natural buoyancy, the resistance to a high light regime, a relatively low growth rate [14] and the ability to utilize a wide range of organic phosphorus compounds [15]. Under calm conditions, filaments accumulate as surface blooms that are often large enough to be detected by satellite. *Trichodesmium* bloom production supports a large significant heterotrophic community, important for carbon and nitrogen fluxes and remineralization [14]. Some evidences of *Trichodesmium* spp. toxicity are available, related to more than one compound. Ramos *et al.* [16] found 0.1-1 μg g^{-1} microcystins (MCs) in a bloom of *Trichodesmium erythraeum*, which was recorded for the first time in 2004 in the Canary Islands Archipelago, during the warmest period recorded since 1912. Samples from a *Trichodesmium erythraeum* bloom off the Brazilian coast were analyzed for MCs, saxitoxins analogues and cylindrospermopsin [17]. MCs and STX were found in the range 10-302 μg g^{-1} MC-LR equivalent and 2-10 μg g^{-1} STX equivalent, respectively; samples resulted toxic to sea-urchin larvae, but did not show any acute toxicity in the mouse bioassay. *Trichodesmium* has also been known to produce the same toxins isolated from contaminated fishes involved in the ciguatera fish poisoning, an important food borne intoxication due to ingestion of fish contaminated with ciguatoxin [18]. Toxicological tests on lipid and water-soluble extracts from New Caledonia lagoon *Trichodesmium* spp. confirmed the production of ciguatoxin-like and neurotoxic-paralytic activity (PSP like toxin) [19]. Recently, the neurotoxic palytoxin and 42-hydroxy-palitoxin have been isolated and characterized from *Trichodesmium* bloom in New Caledonia [20]. The Authors suggest that the ingestion of fish contaminated by both palytoxin and ciguatoxin-like could lead to intoxication suffered from New Caledonian population. A novel cytotoxic toxin, trichotoxin, has been characterized from *Trichodesmium thiebautii*, from the Gulf of Mexico [21].

The planktonic species *Synechococcus* is ubiquitous in the oceans and nutrient rich marine environments, except for Arctic and Antarctic seawater [22]. It occurs in coastal areas and in the river plumes, where nutrients are abundant and salinity lower, together with *Prochlorococcus*, which is usually more abundant, except in upwelling areas. Few studies addressed toxicity in *Synechococcus* and *Synechocystis*. Analysis of crude and partially purified extracts from the two species from several Portuguese coastal sites revealed variable toxic effects on some marine invertebrates (*Artemia*, sea-urchin) [23]. High rate of migratory birds mortality in the Salton Sea, a lake that experiences a range of salinities from brackish to hypersaline in Southern California, has been at least partially associated to the presence of MCs produced by *Synechococcus* [24]. Recently, the presence of MCs in water and their accumulation in mussels in the Amvrakikos Gulf (Greece) was associated to the presence of *Synechococcus*-*Synechocystis* cells, as the only cyanobacteria in the analyzed community [25]. These results suggest that the risk associated to cyanobacteria in the seawater is probably not restricted only to filamentous species, but can be extended also to ubiquitous species like *Synechococcus*.

Coastal zone and brackish areas

The distribution of *Microcystis* which is a well known toxic freshwater genus, has now spread into several estuaries, with possible consequences on fishery production [26]. Indeed, *Microcystis* shows some salinity tolerance, with no significant variation in growth rate and toxin per cell quota up to 10 g salt L^{-1} [27]. *M. aeruginosa* can resist and produce toxins for one week to sudden exposure to salinities up to 17 g L^{-1}, although cell lysis causes an increase in extracellular quota of toxins of about 30% [27]. As reported in Funari *et al.* [8], heavy rainfall and floods have an important role in transporting cyanobacteria in estuaries and coastal waters, where many aquaculture plants are located, thus increasing the risk of exposure of edible organisms.

Toxic *Microcystis aeruginosa* and not toxic *M. wesenbergii* have been recently found in a shallow brackish lake (0.9% salinity) in China, following rising eutrophication conditions in the last few years, together with *Aphanizomenon* and *Anabaena* [28]. The brackish strains are not genetically different (16S rRNA, *mcyB*) from surrounding freshwater lakes, suggesting that the diffusion of *Microcystis* in the brackish waters is a recent event, and/or that the lake salinity does not exert a strong evolutionary pressure [28]. Even if the specific content of detected MCs in these strains is at present significantly lower than in other freshwater strains, it can become a future health problem at increasing densities/frequencies of blooming, since the basin represents an important fishery base [28].

Within Breton Sound estuary, Louisiana, during one year study (Sept 2007-Aug 2008) aimed to observe the effects of high and low river inputs on the phytoplankton community of the estuary, 5 toxin producing genera of cyanobacteria were observed, which were at least an order of magnitude more abundant than 4 genera of toxin producing dinoflagellate. The cyanobacteria genera included *Anabaena, Anabaenopsis, Microcystis, Raphidiopsis* and *Cylindrospermopsis*; they were most frequently observed in the outer portion of the estuary when the river input was low, and most abundant during warm season and at low nutrient concentration. Particulate and dissolved MCs were almost always detected throughout the estuary, in a range of concentrations 0.10-2.92 μg l^{-1} [29].

The northern reach of San Francisco Bay has been infested by an extensive bloom of the colonial form of Microcystis aeruginosa in 1999, and again in 2003, when 180 km of connected waterways was also covered by a bloom. Lower salinities associated with higher temperatures favored the high cyanobacterial density [30], whose origin was attributed to resident cells in sediment and seeded cells from tributaries and high streamflow. Microcystins were found along the waterways, with the highest concentrations in the transition zone, between fresh and brackish water, and in samples of zooplankton and clams, at levels of 0.7-3.5 μg g-dw^{-1} in the former and 0.02 μg g-dw^{-1} in the latter.

Benthic cyanobacteria

Besides planktonic cyanobacteria, benthonic species have also been described, producing cyanotoxins, although mechanisms and factors influencing their production are much less known than for planktonic species.

Cyanobacterial mats have been found in hot springs, a habitat characterized by harshness and extreme conditions, in which they can survive due to genetic adaptations [31]. Since most hot springs are accessible to the public worldwide, as recreational places for citizens and tourists and some are also used as cooking resources by rural communities, human exposure can easily occur. In a study of several hot springs in Saudi Arabia, 12 out of 17 isolated benthic species possessed lipopolysaccharide endotoxins (LPS) and 2 of them, *Oscillatoria limosa* and *Synechococcus lividus*, produced MCs at concentration ranging from 468 to 512.5 μg g^{-1} [32]. Dissolved MCs (5.7 μg l^{-1}) were also found in water. In hot springs on the shore of the alkaline Lake Bogoria in Kenia, dominated by *Phormidium terebriformis*, *Oscillatoria willei*, *Spirulina subsalsa* and *Synechococcus bigranulatus*, MCs and ATX are considered a possible cause for the deaths of Lesser Flamingos [33].

Toxic benthic species are well represented also in freshwater environments. Two southeast Queensland populations of *Lyngbya wollei*, a common freshwater mat-forming species, have been found to produce CYN and deoxyCYN at significant level [34], whereas in Southern United States the same species is known to produce STX [35]. In California some unidentified benthic filamentous cyanobacteria were isolated from four drinking water reservoirs, producing high concentration of MC-LR per carbon unit (1.15 to 4.15 μg mg^{-1} C^{-1}) [36]. Several toxic species of cyanobacteria (*Anabaena subcylindrica, A. variabiles, Calothrix parietina, Nostoc spongiaeforme, Plectonema boryanum, Phormidium corium* and *P. tenue*) were isolated from mats in the Nile River and in irrigation canals, producing MCs [37]. Sediments in Nile River and irrigation canals were also found to be contaminated by *M. aeruginosa* and MCs, suggesting a risk for the benthic fauna [38].

Anatoxin-a producing benthic species such as *Phormidium* sp. and *Oscillatoria* sp. have been identified as the causative agent of dog killing in different countries [39-42].

Benthic cyanobacteria are widespread also along maritime coasts, often forming visually epilithic growths and mats on rocks and sediments. The intertidal zone is dominated by taxa of filamentous non-heterocystous *(Lyngbya, Microcoleus, Phormidium, Schizothrix)* and heterocystous *(Rivula, Calothrix, Scytonema)* genera. The benthic coastal filamentous *Lyngbya maiuscula*, widespread in subtropical and tropical estuarine and coastal waters, is well known for the production of a variety of biologically active components, many of which highly toxic [43].

In the infra-littoral zone, a high biodiversity is found, especially near coral reefs, where the *Oscillatoria*-ocean

genera *(Oscillatoria, Hydrocoelum, Microcoleus)* are represented by many species. Recently, the production of homoanatoxin-a has been reported from mats of *Hydrocoelum lyngbyaceum* in New Caledonia, where giant clams intoxication has been reported, suggesting a possible relationship between the episodes and the presence of the neurotoxic cyanobacterium [44].

HUMAN HEALTH
EXPOSURE AND EFFECTS
Toxicological profiles of cyanotoxins

The presence of a cyanobacterial species or its blooming is not always synonymous of toxicity, which is rather associated to the ratio toxic vs non-toxic strain within the population and to dynamics through which this ratio is able to change in a very short time. A combination of temperature, light and nutrients, can affect, although with still unknown pattern and specific for each species, not only the success of toxic vs non-toxic strains and hence the total production of cyanotoxins in quantitative terms, but also the profile of cyanotoxin variants, which can be even more relevant from a toxicity perspective, considering their diverse toxicological potential [8].

A detailed description of the toxicological properties of cyanotoxins known so far can be found in extensive publications [1, 2, 6]. In the following a brief outline of their toxicological profile is depicted.

Among cyanotoxins, the most investigated group is that of hepatotoxins, which includes about 100 different congeners of cyclic epta-peptides termed microcystins (MCs), differing from each other for amino-acids substitutions in position 2 and 4 and other changes such as methylation/desmethylation. Microcystin mechanism of action is associated with specific inhibition of protein serine/threonine phosphatases (PP1 and PP2A), altering phosphorilation of cellular proteins involved in signal transduction [45]. Each congener is characterized by a different acute toxic potential and indeed, LD_{50}s are spread in a wide range of values (from 50 up to 1200 µg/kg) [1]. Recently, besides hepatotoxic effects, neurotoxic potential has been assessed for MC-LF and to a lesser extent to MC-LW and MC-LR [46]. The long-term effects, known for MC-LR, include tumour promoting activity, due to which IARC has classified MC-LR as possible human carcinogen (class 2B) [47]. In the absence of information on the other congeners, on the basis of the highest acute toxicological properties of MC-LR, concentration MC-LR equivalents are usually used as default value for the total concentration of all MC variants [2]; however, the extrapolation from the acute toxicity ranking among MC congeners to the chronic toxicity remains to be demonstrated.

Microcystins are produced mainly by *Microcystis* spp., *Planktothrix* spp. and some *Anabaena*. Within the same species, only some strains possess the gene for MCs biosynthesis: this is quite relevant since the toxicity of a cyanobacterial bloom is determined by its strain composition, *i.e.*, the relative share of toxic vs nontoxic genotypes: remarkable and rapid variations of the genotype ratio can occur even on a weekly scale [48]. In addition, it has been demonstrated that a mechanism of up- and down-regulation related to cell densities can regulate toxin production, so that the rate of toxin production can change in few hours [49]. Therefore MCs concentrations in water can significantly vary as the result of cyanobacterial population dynamics. The 6 variants of the penta-peptide nodularin (NOD), produced by the brackish-water species Nodularia spumigena, share the major toxicological feature with MCs.

Neurotoxins (*e.g.* anatoxins, anatoxin a(s) and saxitoxins), are produced by strains of the genera *Oscillatoria, Phormidium, Aphanizomenon* and *Anabaena*. All of them act on the neuromuscular system by blocking skeletal and respiratory muscles, causing death by respiratory failure. Anatoxins (ATX) are potent pre- and post-synaptic depolarizing agents, efficiently competing with acethylcoline for nicotinic receptors in neuromuscular junctions and in the central nervous system [50]. Anatoxin-a(s) irreversibly inhibits acetylcholinesterase (AChE) in the neuro-muscular junctions in the peripheral nervous system only. Saxitoxins (STXs) are a family of more than 30 natural alkaloids, synthesized by both freshwater cyanobacteria and by marine dinoflagellates by similar processes [51]: they block Na-channels in neuronal cells [52] and Ca^{++} and K^{+} channels in cardiac cells, thus preventing the propagation of electrical transmission within the peripheral nerves and skeletal or cardiac muscles [53]. Based on the kind of intoxication, they are also called Paralytic Shellfish Poisoning (PSP).

Cylindrospermopsin (CYN) is produced by *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, *Umezakia natans*, or *Raphidiopsis curvata*. Its main target organs are the liver and kidney [54, 55], but its citotoxicity is common to other organs. Cylindrospermopsin has a late and progressive acute toxicity, associated to protein synthesis inhibition [56]. However, its metabolites, which have not been identified so far, very likely act with a different mechanism, involving interactions with DNA [57], as supported by CYN induced *in vitro* DNA damage in mouse primary hepatocytes [58] and in HepaRG cells [59].

In spite cyanotoxins represent an emergent health problem, the available toxicological information are limited, data on humans are very scant, and risk assessment is possible only in few cases, with huge degree of uncertainties [1]. In order to improve the reliability of quantitative risk assessment to protect human health, new toxicological information should become available. USEPA has indicated the study of relative contribution of different exposure routes to total exposure, the role of metabolism in toxic responses and detoxification, and particularly, the characteristics of human toxicokinetics, as major research needs [60]. Indeed, species-specific differences are very often attributable to differences in toxicokinetics processes, affecting the internal dose.

Other possible factors modulating the outcome of cyanotoxin exposure in humans are linked to differential exposure, or to the involvement of active transporters or enzymes catalysing cyanotoxin metabolism, characterized by genetic polymorphism, such as in the case of MC conjugated by human glutathione-S-transferases [61]. This could end up in different levels of expression and enzymatic activity within the human population, suggesting the presence of groups of people differently susceptible to cyanotoxin-induced effects.

Human health

Human exposure scenarios and hence health impacts are strictly related to the use of water bodies, generally associated to source of drinking water or for haemodyalisis purposes and recreational activities; the human exposure can be also indirect due to consumption of freshwater fish, crops and vegetables, or items of animal origins following the use of contaminated water for irrigation or in farming activities. Therefore appropriate monitoring programs (for either water and consumer products) should be planned and adopted depending on the cyanobacteria specie, the characteristic and the use of the water body, foreseeing intensification of controls after rain falls and floods, water evaporation associated to higher temperature and drought, and to thermal stratification, all factors expected to favor cyanobacterial bloom (Funari *et al.* 2012).

1. Drinking and recreational waters

These two sources for cyanotoxins human exposure have been considered from the very beginning and guidelines values have been set by WHO [62, 63] and risk assessment carried out [1] on the basis of toxicological information available for the most studied cyanotoxins. WHO defined guidance values have been adopted by some countries as specific regulatory framework or recommendations.

Episodes of acute/short term human intoxications due to drinking water consumption have been reported in some countries as a consequence of failure or inefficiency of water treatment [64]. Gastroenteritis and liver damages were among the most frequently reported diseases, although other effects have been reported as well. The chronic risk associated with repeated exposure to cyanotoxins in humans through drinking water is difficult to be demonstrated, as information from epidemiological studies is scarce and inconclusive. Indeed, the International Agency for Research on Cancer (IARC) concluded that it was not possible to associate the excess of hepatocellular carcinoma and of colorectal cancer reported in the available epidemiological studies with exposure to MCs [47].

In freshwater recreational settings, the available data show a range of diverse symptoms associated with exposure to cyanobacteria, like severe headache, pneumonia, fever, myalgia, vertigo and blistering in the mouth [1]. Some allergic responses to cyanobacteria have also been published, possibly due to the action of cyanobacterial LPS endotoxins [65]. On the basis of available data, it seems possible to conclude that the risk of severe effects for bathers posed by ingestion of cyanotoxins is significant only when cyanobacteria bloom or form scums. Inhalation of aerosol is another possible route of human exposure. In a study, in which aerosols were collected using high and low volume air samplers for 4, 12 and 24 h, close to two lakes in New Zealand, experiencing blooms of *Nodularia spumigena* and *Microcystis*, levels of MCs (1.8 pg/m^3) and nodularin (16.2 pg/m^3) detected in the air did not appear to represent an acute or chronic hazard to humans [66]. However, aerosolized toxins should be considered when developing risk assessments for lakeside populations and recreational users where inhalation of cyanotoxins may be a secondary exposure source to a primary oral exposure [66].

2. Haemodyalisis

The most serious known episode associated to human exposure to cyanotoxins occurred in Brazil, where 56 out of 130 haemodialysed patients died after treatment with MC contaminated water [67]. Again in Brazil, in a later survey 0.2-0.96 μg/L MC were found in plasma of apparently asymptomatic patients following a bloom in the water body supplying some dialysis centers [68].

This route of exposure is probably underestimated over the world: indeed, the quality of water used for hemodialysis is not subjected to any mandatory regulations in most countries and cyanotoxin detection is not requested as a routine quality control. In addition, results obtained in Brazil revealed that the reverse osmosis system compulsory used in that country, did not prevent MC contamination of water used in the treatment of dialysis patients, although different reverse-osmosis membranes have been described as very efficient, showing retention rates in the range 96.7%-99.9% for two MC variants [69]. This could be tentatively attributed to the fact that the filters in the system are usually set to properly work at 20 °C ± 5 °C, operational temperatures that especially during summertime can be easily exceeded in many countries.

3. Fish, shellfish, and molluscs

No cases of human intoxications associated with consumption of aquatic organisms contaminated by cyanotoxin have been reported so far; however, this possibility does exist, since the number of reports about the presence of cyanobacteria in coastal and brackish waters is increasing. And indeed, the major emerging problem with respect to human exposure might come from transitional-, brackish- and coastal sea-waters, which are going to be strongly affected by increasing heavy rainfall and floods [8]. Edible organisms will be more often exposed to cyanotoxins which are not routinely monitored, according to the existing international and national regulations. This

pattern of exposure might be relevant particularly for edible aquatic organisms grown in aquaculture plants which are preferentially located in lagoons, brackish- and estuarine-waters.

Significant levels of cyanotoxins have been detected in aquatic organisms: maximum concentrations of 370, 2700 and 16 000 μg/kg have been reported in the edible parts of fish, crustaceans, and mussels, respectively [1]. Juvenile rainbow trout has been experimentally found to accumulate, in the whole body, anatoxin-a with a bio-concentration factor (BCF) of 30-47, based on fresh weight [70]. Fish viscera and the hepatopancreas of shellfish and mollusks usually contained higher levels than edible parts [71]. Significant levels of MCs have also been found in the tissues of the demersal blue crab Callinectes sapidus (up to 820, 65 and 105 μg MC/kg in hepatopancreas, viscera and muscle, respectively), during a bloom of toxic cyanobacteria in the hypereutrophic Lac des Allemands, that is the end-member of the Barataria estuary (Luoisiana) [72].

Marine cyanobacteria such as *Nodularia spumigena*, *Aphanizomenon flos-aquae*, and *Anabaena* sp. bloom widely in the Baltic Sea; in addition some other cyanobacteria like *Trichodesmium* are now described in temperate sea water, favoured by poleward shift [8]. Since cyanotoxins can accumulate in aquatic organisms tissues, and sea-food is not routinely monitored for cyanotoxins presence, the dietary exposure of consumers through sea food may be particularly relevant considering the size of the exposed population, and the reported levels of contamination. A recent survey of literature data on biomagnification factors of MCs, showed that biodilution (BCF < 1) is the main process in aquatic environment for all primary consumers except for zooplankton and zooplanktivorous fishes, thus highlighting the importance of fish diet in their relative ability to bioaccumulate cyanotoxins [73]. BCF is also related to the length of exposure, which makes more relevant the problem in environments where toxic organisms are constantly present [73]. Recently, the death of 21 sea-otters in Monterey Bay National Marine Sanctuary has been associated with the trophic transfer of MCs flown from three tributaries into the Ocean [74]. The authors found compelling evidence that the mammals death was caused by accumulation of the hepatotoxins through food web magnification. The same can occur in the human diet.

MCs have been found in marine waters and in *Mytilus galloprovincialis*, at a concentration ranging between 45 to 140 ng g^{-1} of whole mussel, during a one year monitoring program in the Mediterranean waters of the Amvrakikos Gulf (Greece) [25]. A maximum level of 105 μg MC/kg has been reported in the muscle of the blue crab *Callinectes sapidus*. Considering a daily consumption of 200 g meat in a meal, approximately 20 μg MC/kg bw would be ingested, which is comparable to the reference values for subchronic risk of 24 μg/kg bw per day for an adult weighing 60 kg [1]. This suggests the need of a more comprehensive data base related to potential human exposure in order to estimate the actual threat for human health from this source. The population living in the coastal areas or close to aquaculture plants, likely being strong seafood consumers, can be considered the most vulnerable ones and cyanobacteria pose a serious threat for the future.

Furthermore, in coastal waters (including estuarine and brackish areas), toxins produced by cyanobacteria can contaminate sea-food already exposed to toxins released by other marine organisms (*i.e.* harmful algae). Particular attention has been given so far to STX, since their presence in seafood has caused several episodes of severe intoxications and deaths in human beings, as reported in a comprehensive FAO Report [75]. Human fatalities due to ingestion of STX-contaminated seafood still occur in countries where prevention programs are not effectively implemented [76] and it has been estimated that more than 2000 human cases of food-borne PSP occur globally every year with a mortality rate of 15% [77]. The problem is not limited to STX, indeed sea-food can be contaminated by toxins produced by cyanobacteria and by tropical/subtropical species of harmful algae, which have been subjected to a similar poleward movement to temperate areas. The production of palytoxin-like compounds and 42-hydroxypalitoxin by *Trichodesmium* spp. in coastal areas where the presence of *Ostreopsis ovata* (producing the same toxins) has also been described is only one example. In addition different toxins have a similar mechanism of action (as MC and okadaic acid, both inhibitor of PP2A) and hence a possible interaction for toxicity is another aspect that deserves further consideration.

4. Products of animal origin and vegetables

Another possible impact on human health is due to residues in edible products of animal origin. Data available seem to indicate that repeated MC ingestion (up to 10 μg/L contained in an extract of *M. aeruginosa*), not toxic for cows and sheep, correspond to negligible residues in meat, milk and dairy products [78, 79]. Therefore, the risk associated to their consumption has not been considered significant. However, this seems to be valid for hydrophilic MC variants, which have a low potential for bioaccumulation, but information related to lipophilic MC congeners (likely showing a different elimination kinetics in the animal) or to other cyanotoxins are not available. Finally the possible consumption of animal viscera (liver and sometimes intestines), which is often neglected in exposure scenarios but frequent in some geographical areas or ethnic groups, can represent a problem which deserves special attention, due to the high levels of MCs which can be expected in these organs. Specific data on these issues are missing so far.

Consumption of vegetables, irrigated with contaminated waters, could represent an additional source of human exposure to cyanotoxin. It has

been reported that some vegetables can retain MCs present in irrigation water infested by blooms or scums, where the cyanotoxins levels was so high to be able to inhibit the plant growth and induce phytotoxicity [80, 81], lowering seed germination and seedling growth [82]. Detrimental effects on plants growth were observed also when plants were exposed for 30 consecutive days to water containing 0.5-4 μg L^{-1} MC-LR equivalents [83]. This poses a threat to the yield and quality of crops having an impact on food availability, but at the same time strongly limits the possibility for human exposure. The low concern posed by human exposure to cyanotoxins via vegetable consumption is supported by the observation that when watering broccoli and mustard seedlings with water containing MC concentrations typically found in natural surface waters (1-10 μg/L), the toxins were found only in the roots, at levels of no human health concern [84]. No information on other MC variants or cyanotoxins is available for the time being.

5. Food supplements

Food supplements, known as blue-green algae supplements (BGAS) are available on the market and over the internet in a variety of forms: tablets, powder, capsules consisting of extracts or dried powder, mainly derived from *Spirulina* spp. and *Aphanizomenon flos-aquae* grown in artificial ponds or collected directly from the natural environment. Producers claimed they are health-promoting natural products, used as a support in losing weight during hypocaloric diets, increasing alertness and energy and elevated mood for people suffering depression and for their supposed anti-inflammatory, anti-bacterial, anti-viral, anti-cancer, hypocholesterolemic and hypotriglyceridemic properties and immune-stimulating functions [85-87]. In addition, BGAS are administered to children as an alternative, natural therapy to treat attention deficit hyperactivity disorders (ADHD) [88]. No clear comprehensive evaluation of the putative benefic effects resulting from their assumption has been carried out yet.

Spirulina sp. and *A. flos-aquae* can coexist with other potentially toxic strains of cyanobacteria which share the same habitat, as *Microcystis* sp.; the contamination of BGAS sold as nutraceuticals in different countries with MCs has been repeatedly shown [89-92], in many cases showing a substantial percentage of samples (approximately 40-70%) with MCs levels up to 35 μg/g per dry weight product [93], thus exceeding 1 μg/g MC-LR equivalents, the provisional guidance value set by the Oregon Dept. of Agriculture, starting from a provisional TDI of 0.04 μg/kg bw per day [63]. The presence of DNA belonging to *Microcystis* genus in all *A. flos-aquae*-derived contaminated samples, strengthens the hypothesis that *M. aeruginosa* harvested together with *A. flos-aquae* is responsible for the contamination [92].

Assuming a daily consumption of 4 g of BGAS, easily achieved through a consumption of 4-8 tablets/day, and a contamination level of 1 μg/g, a daily intake of 4 μg MCs/person would result, which would be almost twice as high as the TDI value, posing a risk for the chronic consumer, independently from exposure to MCs from other possible sources, as contaminated water or fish. For moderate users, taking pills for a limited period of time, the risk would be relevant at higher levels of contamination, which on the other hand are quite frequent, as mentioned above.

The estimate of the actual exposure is difficult, since BGAS are perceived as safe 'natural' products and are consumed following individual programs, without any prescription nor indication for a specific daily dosage. Indeed, consumption of extremely high daily doses (up to 20 g) has been reported [93], for which even the possibility of acute hepatotoxicity can be expected at levels of contamination around 5 μg/g MC-LR equivalents [92].

To date neurotoxins have never been detected in BGAS [92, 94]; the presence of other cyanotoxins such as cylindrospermopsin has also been excluded [95], but data are scant.

ANIMAL HEALTH

Several poisoning episodes of livestock, wild and domestic animals have been associated with the occurrence of cyanobacterial blooms in surface waters used for drinking [96]. Although anecdotal reports dated many years ago, the issue has been raised as relevant only more recently, when a specific cause-effect relationship could be established, due to appropriate sampling schedules of both water and body fluids and investigative chemistry techniques.

Animals, especially caws and sheep can be exposed to extremely high levels of toxins in the presence of scums accumulating by lake or river side: in this condition they can ingest lethal doses contained in water volumes lower than their daily consumption. In addition it has been reported that animals of different species seem to drink preferentially waters contaminated by high cyanobacteria density rather than clean ones [97]. Cattle murrain, more than one hundred calves and heifers, has been documented in Switzerland alpine pastures [98]. The animals died by acute hepatotoxicosis, but presented also symptoms of neurotoxicity. The analysis of the highly oligotrophic water bodies in the pasture, showed an abundant community of mat-forming cyanobacteria, dominated by *Oscillatoria limosa* and *Oscillatoria tenuis*, and MCs were detected both in the mats and in the water [98].

A case of sheep mortality associated with PSP from the cyanobacterium *A. circinalis* has been reported in South Wales (Australia) [99]: the toxin was present in the small intestine of the dead animals. Interestingly, the toxin profile of the environmental sample indicated a high content of C-toxin (70%), a PSP variant with low toxicity, whereas in the small intestine the more toxic gonyautoxin was the dominant form (87%), clearly confirming the possibility of biotransformation within the organism between PSP forms with different toxicity.

Diagnosis of ATX and HTX poisoning in dogs after drinking and bathing in infested waters have been reported from Scotland [39], France [40], Ireland [41], and in the USA [42]. In most cases, dog poisoning was associated with neurotoxic cyanotoxins produced in rivers by benthic taxa, such as *Phormidium* sp. and *Oscillatoria* sp. These species readily form biofilms, attached as a sticky mass to surfaces, including dog hair during bathing. Exposure of dogs may be significantly increased by fur licking after immersing in a bloom.

Anatoxin-a(s) has been frequently associated with mass mortality of birds [100]. Anatoxins and MCs are also considered a contributing factor in the deaths of Flamingos in Kenia [33].

Besides the ethical and ecological problems related to animal welfare and protection, even when wild animals including birds are considered, such events have also economic consequences (*i.e.* on farming activities).

DATA GAPS AND RESEARCH NEEDS

In assessing the risk for the exposed population, the wide-spreading of cyanobacteria in environments other than freshwater as described above indicate that additional focus should be given to the exposure to toxins via food web, due to the expansion of cyanobacterial population in coastal and brackish waters, where many aquaculture plants are located. This scenario is likely to become more important, for the additional effects of climate changes, specifically the increase in frequency and intensity of heavy rainfall and floods [8].

Other emerging scenarios requiring additional focus, by characterising the levels of exposure in a more accurate way, are the case of haemodialysis, food supplements consumption, mat forming and marine cyanobacteria.

The possibility of changes in the toxicological profiles of the toxins produced by some species, under environmental stress, is an additional issue for potential concern. Indeed, both *C. raciborskii* and *Trichodesmium* species have been found to produce different toxins with different, and often unknown, toxicological profile, depending on the geographical areas: this is particularly important for these two subtropical species, due to their spreading into temperate regions, associated to their poleward shift [8]. As another example *Limnothrix redekei*, which is generally reported to produce MCs, is also able to produce another still unidentified water soluble toxin as it causes *in vitro* and *in vivo* effects different from those previously described for known cyanobacterial toxins [101, 102]. Since the genus *Limnothrix* occurs in a range of freshwater habitats that are used as sources of drinking water, the toxicological and chemical characterization of the toxin is an urgent need.

Recently, a research on benthic and pelagic cyanobacteria from Portuguese coasts, revealed that isolates of *Leptolyngbya*, *Oscillatoria* and *Phormidium*, as well as *Cyanobium* and *Synechococcus*, induced acute toxicity in nauplii of the brine shrimp *A. salina* [103]. Apparently, none of the known toxins has been detected, even if fragments of *mcyE* gene have been found in several isolates, indicating the possibility of new toxins produced by these organisms. The potential risk associated to these and other cyanobacterial species producing unknown toxins is of particular concern for human health, and suggest the need for unspecific biological methods to detect the potential toxicity of a bloom, not just linked to analytical detection of known molecules.

Furthermore, in assessing the risk, aggregate/cumulative exposure has to be considered, for those individuals that can be exposed to the same cyanotoxin via different routes of exposure (*i.e.* drinking water, contaminated food and food supplements) or to different toxins at the same time. Combined exposure to different cyanotoxins represents very likely the rule rather than the exception, since the same cyanobacteria may produce more than one toxin as in the case of MC variants and STX derivates, characterized by different toxicity in addition to the fact that blooms are very rarely characterized by the presence of a unique cyanobacterium. Furthermore, cyanobacteria can produce other types of biologically active peptides, such as cyanobactins, on which research has just started. These are small cyclic peptides recently found in cyanobacteria and reported to have pharmacological activities (*i.e.* antimalarial, antitumor) and produced through the proteolytic cleavage and cyclization of precursor peptides [104].

A model to predict the combined neurotoxic effects of binary and ternary mixtures of STX has been proposed, indicating that the most potent toxin is by far the most relevant component, whereas the less toxic derivatives should be order of magnitude more concentrated to contribute to the cumulative toxic potency [105]. In the case of MC variants, exposure is generally represented by a mixture of MC congeners: usually by adopting a conservative approach, acute toxicity is referred to MC-LR equivalents, since MC-LR is the most acutely hepatotoxic (when administered ip). An approach similar to the "toxicity equivalent factor" (TEF), used for polychlorinated dibenzo[p]dioxins (PCDD), has also been proposed for MCs and NODs [1, 106]. However, such an approach is generally based on data obtained after intraperitoneal treatment, which is poorly representative of the actual human exposure conditions, it is limited to acute toxicity and does not consider the possibility that some variants could also have targets other than the liver, such as the case of neurotoxic potential shown by LC-LF, and as a consequence should be used with caution.

Although accumulation of different cyanotoxins up to significant levels in aquatic organisms usually eaten by humans could be particularly relevant (especially for neurotoxins), the issue of exposure to cyanotoxins' mixture has not been sufficiently inves-

tigated so far. In coastal environments cyanotoxins will sum up to algal toxins, increasing the risk of intoxication of the exposed population, as described above for *Trichodesmium*.

Moreover, concomitant exposure with other chemicals may be of relevance, such as in the case of organophosphorus pesticides. Indeed, exposure to the insecticides could potentiate the ATX-s induced toxicity by inhibiting AChE activity also in the brain, not only in peripheral nervous system [107]. Combined and single effects have been reported associated with the exposure of pesticide carbaryl and toxic *Microcystis aeruginosa* on the life history of *Daphnia pulicaria* [108].

In addition to co-exposure among chemicals, rivers and lakes affected by cyanobacterial mats might promote the growth of autochtonous pathogens and pathogenic microorganisms like *Legionella*, which has been shown to use algal extracellular products as carbon and energy sources [109] and can proliferate in biofilm, in association with amoebae or protozoa or cyanobacteria [100]. This would increase the possibility of transmission of the related infections. This is also true for brackish-/sea-water, where heterotrophic bacteria communities associated with cyanobacteria include possible pathogenic taxa that need to be considered in assessing the risk for human health. Berg [111] found that most (90%) of the *Aeromonas* spp. associated to cyanobacteria in Baltic Sea, were positive for at least one of several factors of virulence. Blooms of the toxic cyanobacterium *Nodularia spumigena* are an ideal growing medium for several pathogenic microorganisms, including the deadly serotypes of *Vibrio cholerae* O1 and O139, and *V. vulnificus* [112], exposing people also during bathing activities. Decaying blooms of *Microcystis aeruginosa* can increase the concentration of *Vibrio* spp. in estuarine and brackish environment [113]. Short term experiments demonstrated that pathogenic serotype *Vibrio* O139 can survive in saline microcosms in association with *Anabaena, Nostoc* and *Hapalosiphon* spp. [114].

Important open research fields and data gaps are related to new scenario of exposure and to the potential bioaccumulation of cyanotoxins in the food web, as well as to new patterns of co-exposure between cyanotoxins and anthropogenic chemicals and/or algal toxins or pathogenic microorganisms. However, data gaps are also present in the elucidation of dynamics affecting blooming and production of different cyanotoxin variants and the environmental factors able to influence these processes. The environmental factors responsible on a short time scale for sudden changes in the toxin genes expression, or on longer time scale for the dynamic of toxic/non toxic individuals; the physiological/ecological role of cyanotoxins production and factors triggering the active transport outside the cell and the interrelationship between cyanotoxins and other biologically active peptides produced by cyanobacteria, are specific fields of research particularly needed to better predict the occurrence of blooms and their toxicity, and consequently to identify efficient prevention measures. In addition, many data gaps are also present in the characterization of the toxicological profiles, especially regarding long term effects. The study of these issues represents a key step for a better risk assessment and management, allowing the preparation of appropriate and efficient plans for prevention and human health protection.

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Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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Waterborne outbreaks of cryptosporidiosis

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Abstract. Water is the most commonly reported vehicle of transmission in *Cryptosporidium* outbreaks. While mains drinking water quality is highly regulated in industrialised countries, treated recreational water venues remain highly variable and these have emerged as important settings in the transmission of cryptosporidiosis. Epidemiological investigations of outbreaks benefit from supplementary microbiological evidence and, more recently, the application of molecular typing data to link isolates from cases to each other and to suspected sources. This article documents how waterborne *Cryptosporidium* outbreaks are identified and reported, how such outbreaks have acted as drivers of regulatory change, and some of the recent developments in the detection and investigation of these outbreaks and their spread, especially the application of molecular typing assays.

Key words: Cryptosporidium, genotyping, recreational, drinking, water.

Riassunto *(Epidemie di cryptosporidiosi trasmesse con le acque)*. L'acqua è il più comune veicolo di trasmissione di epidemie dovute a *Cryptosporidium*. Mentre la qualità delle acque potabili distribuite da acquedotti è fortemente regolamentata nei paesi industrializzati, la qualità delle acque trattate di ambienti ricreativi chiusi è fortemente variabile e questi ambienti si sono rivelati importanti nella trasmissione della criptosporidiosi. Le indagini epidemiologiche sulle epidemie traggono beneficio dalle evidenze microbiologiche e, più recentemente, dall'applicazione dei dati di tipizzazione molecolare per collegare i ceppi isolati con i casi d'infezione e con le fonti di contagio sospette. Quest'articolo documenta come vengono individuate e notificate le epidemie di *Cryptosporidium* trasmesse con le acque, come tali epidemie hanno agito da guida per migliorare la normativa, ed alcuni recenti sviluppi nella rilevazione e nelle indagini di queste epidemie e della loro diffusione, in particolare l'applicazione dei saggi di tipizzazione molecolare.

Parole chiave: Cryptosporidium, genotipizzazione, acqua ad uso ricreativo, acqua potabile.

INTRODUCTION

Human infection with the protozoan parasite *Cryptosporidium* causes the gastrointestinal disease cryptosporidiosis. Of the 25 or so species currently recognised, 15 have so far been reported in humans, of which some are established as human pathogens: *C. parvum*, *C. hominis* (which are the most commonly detected species in human cryptosporidiosis worldwide) and *C. meleagridis* are supported by human infectivity and clinical outcome data from feeding trials in adult volunteers [1-3], *C. cuniculus* (formerly the rabbit genotype) caused a drinking waterborne outbreak in the United Kingdom (UK) [4], and *C. felis* and *C. canis* were associated with diarrhoea in children in a shanty town in Peru [5]. Dose response studies have shown similar ranges for some *C. parvum* isolates compared with *C. hominis*, and small numbers (< 10) of parasites ingested can cause disease [1, 2]. Other *Cryptosporidium* species are rarely reported human infections, or have never been found in humans, and many are considered adapted to farmed animal or wildlife hosts [6] *(Table 1)*.

Transmission is by the faecal-oral route, from either humans or animals, depending on the *Cryptosporidium* species; for example, *C. hominis* has a human infection cycle while *C. parvum* also has susceptible animal hosts causing mainly gastrointestinal disease in young ruminants. The natural host for *C. cuniculus* is the European rabbit *(Oryctolagus cuniculus)* [13] and for *C. felis*, cats and *C. canis*, dogs. Although *C. meleagridis* was originally identified in farmed turkeys [14] current distribution and risk factors for human acquisition are not known; many cases report no contact with birds, the parasite species has a wide host range and other bird-restricted species are not considered a threat to human health. Although some *Cryptosporidium* spp. are highly infectious person-to-person, it is the parasite's ability to survive in the environment and its resistance to chlorine disinfection that support transmission via drinking and recreational waters, and other vehicles such as food. *Table 2* shows the human risk factors for acquisition of *Cryptosporidium* spp. and how these relate to settings where outbreaks have occurred.

Symptoms of cryptosporidiosis, which usually occur between 2 to 12 (usually 5 to 7) days after ingestion of oocysts (the transmissive stage of the life cycle), include watery diarrhoea, abdominal pain, nausea and/or vomiting, low grade fever and malaise, and may last for up to three weeks during which time apparent recovery may be followed by

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Table 1 | *Cryptosporidium species and selected genotypes and their association or not with human cryptosporidiosis*

Cryptosporidium **species**	**Mean oocyst dimensions (µm)**	**Major host(s)**	**Association with human cryptosporidiosis or infection**	**Selected references**
Most commonly associated with human cryptosporidiosis				
C. hominis	4.9 x 5.2	Humans	Common in sporadic cases and outbreaks; infectivity data from experimental infections in adults	[2]
C. parvum	5.0 x 4.5	Humans, ruminants	Common in sporadic cases and outbreaks; infectivity and dose response data from experimental infections in adults	[1]
C. meleagridis	5.2 x 4.6	Homoeothermic birds; mammals including humans	Sporadic cases reported, more frequent in some populations, for example as common as ***C. parvum*** in Peru and Thailand; infectivity data from experimental infections in adults	[3, 6]
Less commonly associated with human cryptosporidiosis				
C. canis	5.0 x 4.7	Dog	Epidemiologically linked to diarrhea in children in a shanty town in Lima, Peru; occasional sporadic cases in various countries, especially developing countries	[5, 6]
C. cuniculus	5.6 x 5.4	Rabbit, humans	Caused a waterborne outbreak in UK; occasional and seasonal sporadic cases in UK, individual reports from France, children in Nigeria	[4]
C. felis	4.6 x 4.0	Cat	Epidemiologically linked to diarrhea in chldren in a shanty town in Lima, Peru; occasional sporadic cases in various countries	[5, 6]
C. ubiquitum	5.0 x 4.7	Various mammals	Sporadic cases in various countries, especially developed countries	[6]
C. viatorum	5.4 x 4.7	Humans	Sporadic cases emerging in UK and Sweden, linked to visits to the Indian sub-continent, South America and Africa	[7]
Rarely associated with human cryptosporidiosis				
C. andersoni	7.4 x 5.5	Cattle	Individual reports from UK, Australia, Malawi	[6, 8]
C. bovis	4.9 x 4.6	Cattle	Individual reports from Australia and India	[8]
C. fayeri	4.9 x 4.3	Red kangaroo	Individual report from Australia	[8]
C. muris	7.0 x 5.0	Rodents	Individual reports from various developing countries	[6]
C. scrofarum	5.2 x 4.8	Pig	Individual report from Czech Republic	[9]
C. suis	4.6 x 4.2	Pig	Individual reports from UK and Peru	[6]
C. tyzzeri (syn. mouse genotype I)	4.6 x 4.2	Mice	Individual report from Czech republic	[10]
Chipmunk genotype I	4.8 x 4.2	Chipmunk; possibly other Sciuridae	Individual reports from USA, France, Sweden	[6]
Horse genotype	4.6 x 4.2	Horses	Individual reports from UK, USA	[11, 12]
Monkey genotype	not reported	Monkey, human	Individual reports from UK, Malawi	[6]
Skunk genotype	not reported	Skunk; possibly other mustelids	Individual report from UK	[6, 11]
Not associated with human cryptosporidiosis or infection				
C. macropodum	5.4 x 4.9	Eastern grey kangaroo	No association	
C. ryanae	3.7 x 3.2	Cattle	No association	
C. wrairi	5.4 x 4.6	Guinea pig	No association	
C. xiaoi	3.9 x 3.4	Sheep	No association	
C. baileyi	6.2 x 4.6	Chicken	No association	
C. galli	8.3 x 6.3	Chicken	No association	
C. fragile	6.2 x 5.5	Black spined toad	No association	
C. serpentis	6.2 x 5.3	Snakes	No association	
C. varanii	4.8 x 4.7	Mainly lizards; snakes	No association	
C. molnari	4.7 x 4.5	Sea bream	No association	
C. scopthalmi	4.4 x 3.9	Turbot	No association	
Various genotypes	Usually within the 4-6 range	Various, or not known if found in environmental samples and no host yet identified	No association	

Table 2 | *Exposure risk factors for human acquisition of cryptosporidiosis, and related outbreak settings*

Risk factor	*Cryptosporidium* species involved	Groups of people at risk	Outbreak settings
Drinking contaminated water	*C. parvum* *C. hominis* *C. cuniculus*	All consumers	Community with mains water supplies Small communities and settings with small or private water supplies
Eating contaminated food	*C. parvum* *C. hominis*	All consumers; people attending events or functions	Community Food establishments (*e.g.* catering establishments, institutions) Specific events (*e.g.* agriculutural fairs)
Traveling to less industrialised countries	*C. parvum* *C. hominis* *C. meleagridis* *C. viatorum*	Sporadic cases; groups of travellers	Various
Recreational activities involving water immersion	*C. parvum* *C. hominis*	Mainly children	Swimming pools Paddling or wading pools Water parks Fountains Natural waters
Contact with farmed animals, especially young ruminants; contact with animal dung	*C. parvum*	Those exposed through occupational and recreational activities (*e.g.* veterinary students, farmers, visitors to petting farms)	Veterinary schools, agricultural colleges, petting farms, farms opened to the public, residential outdoor activity centres
Changing nappies; toileting young children	*C. hominis*	Individual carers and those exposed through occupational activities (*e.g.* nursery/day-care centre employees)	Nurseries; day care centres
Contact with another person with diarrhoea	*C. hominis* *C. parvum* Others – some evidence that all species infecting humans can be transmitted person to person	Individual carers, family members, close contacts	Nurseries, day care centres, institutions such as schools, hospitals, prisons

temporary recurrence [15]. Oocysts may continue to be shed in faeces for many days after symptoms have ceased. About 10% cases may be hospitalised. Treatment is by supportive therapy to prevent dehydration; there is no licenced specific treatment in the EU, although nitazoxanide is licenced by the United States (US) Food and Drug Administration for immunocompetent patients over 1 year of age. Severely immunocompromised patients, especially those with T-cell immune deficiencies, risk chronic or intractable disease and infection of sites other than the gastrointestinal tract [16]. Immune reconstitution is important in these patient groups, as treatment modalities are otherwise undefined, and prevention of infection through risk reduction is paramount. For example, since the late 1990's, the Department of Health in England advised that those with compromised T-cell function should boil all drinking water (including bottled water) [17]. However, whether, given improved drinking water quality and reduction in *Cryptosporidium* risk since then [18], this permanent blanket advice remains necessary there is currently under review. Also of great concern are the longer term sequelae of infection in both the general population and in malnourished children in whom, even following asymptomatic infection, reduced cognitive function and failure to thrive have been reported (reviewed by Putignani and Menichella [19]). Even in non-immune compromised people, there is some evidence to suggest there may be different long term health effects depending on infecting species: for example, those cryptosporidiosis patients infected with *C. hominis* (but not *C. parvum*) were more likely to report joint pain, eye pains, headaches and fatigue in the two months following infection than controls

[20]. The relationship between clinical outcome in terms of severity of acute disease and long term sequelae requires further investigation.

Although people of any age can become infected, most cases of cryptosporidiosis are reported in children under 5 years largely due to intestinal tract immaturity and lack of mucosal immunity [20]. In non-industrialised countries, the peak incidence is in infants less than 1 year old with adult cases rarely identified, and in industrialised countries mainly in 2-4 year olds with a second, smaller peak in adults of child-rearing age. This is likely due to differences in general hygiene and feeding practices for infants, and age-related immunity generated by repeated exposure (for example, low-level exposure through drinking water) in older age groups. However, any immunising effect would come at a high risk to the health of the most vulnerable sectors of the population: young children and immunocompromised patients. The epidemiology of human cryptosporidiosis globally has been reviewed most recently by Putignani and Menichella [19].

The clinical problems of cryptosporidiosis described above contributed to the inclusion of the parasite in the World Health Organisation's (WHO) Neglected Diseases Initiative in 2004 [21]. Further, large waterborne outbreaks have highlighted the clinical and economic importance of *Cryptosporidium*. Waterborne cryptosporidiosis outbreaks are more commonly reported than outbreaks involving other vehicles; up to the end of 2010, a total of 185 outbreaks had been identified and reported globally [22, 23], contrasted with less than 20 foodborne outbreaks [24]. This is only partly because of the features of *Cryptosporidium* favouring waterborne transmission, as some of these also favour the foodborne route:

- multiple hosts for some human pathogenic species, for example, *C. parvum* (mainly humans and young ruminants);
- ubiquitous distribution (*Cryptosporidium* spp. occur worldwide);
- large numbers of oocysts (10^{10}), the transmissive stage, are shed by susceptible hosts during acute infection;
- oocysts are shed containing fully infective sporozoites – no secondary hosts or maturation conditions are required;
- transport vectors may provide further distribution of oocysts within or to the acquatic environment;
- small size of oocysts (4-6 μm for human-infective species) means they may pass between sand grains in filter beds, although other forces including sedimentation and adsorption also interplay during filtration, and application of a coagulant (flocculant) improves removal;
- oocysts can be discharged in sewage effluent in significant numbers;
- oocyst are robust and can survive for months in cool, moist environments; they also survive chlorination and;
- small numbers of ingested oocysts can cause disease [25].

Furthermore, the large scale of some drinking waterborne cryptosporidiosis outbreaks (the Milwaukee outbreak in 1993 involved an estimated 403 000 cases of cryptosporidiosis [26]) led to greater emphasis on monitoring and intervention of water supplies, and greater awareness and investigation of water as a transmission vehicle, including recreational waters such as swimming pools. There is a perception of cryptosporidiosis as a waterborne disease, perhaps at the expense of investigation in other possible routes and investment in their interventions. However, where chlorine disinfection is widely used to treat drinking water, *Cryptosporidium* is indeed one of the most common waterborne pathogens. For example, in a review of 89 waterborne outbreaks of infectious intestinal disease (IID) involving 4321 cases in England and Wales, *Cryptosporidium* was the causative agent in 69% [27]. In recreational waters, *Cryptosporidium* is the leading microbial cause of outbreaks in both the UK and USA [27, 28]. Despite global distribution of *Cryptosporidium*, reports of waterborne outbreaks are weighted heavily towards industrialised countries in the continents of Australia (especially New Zealand), Europe (especially UK and Ireland) and North America [19, 23]. This distribution may reflect national variations in surveillance, reporting, monitoring, and investigation of cases and outbreaks, as well as highlighting risk factors such as intensive stocking of farmed animals, environmental contamination, weather conditions and events, discharge of sewage effluent into drinking water sources, and the use of surface water sources. The pathways and host, parasite and environmental factors that determine the risk of infection, and thus public health outcomes, are shown in *Figure 1*, and how they contribute to waterborne *Cryptosporidium* outbreaks has been reviewed previously [for example, see 19, 22, 29]. Risebro and colleagues used a fault tree analysis to examine the contribution and interpretation of events in outbreaks in the EU occurring between 1990 and 2005 [29]. Of 31 protozoal drinking water outbreaks, 29 were *Cryptosporidium*, and most of these outbreaks were attributed to chronic filtration failures or livestock and rainfall in the catchment, contributing concurrently in 11 outbreaks. Interestingly, the most recent outbreaks in the UK have been attributed to human or wildlife sources *(Table 3)*. It is now possible, using molecular methods, to differentiate the *Cryptosporidium* species found in water samples and supplement catchment data to track the source of contamination to humans, farmed animals or wildlife and to assess the level of risk posed to public health beyond that previously possible from oocyst counts alone.

The aim of this article is to document how waterborne *Cryptosporidium* outbreaks are identified and reported, how such outbreaks have acted as drivers

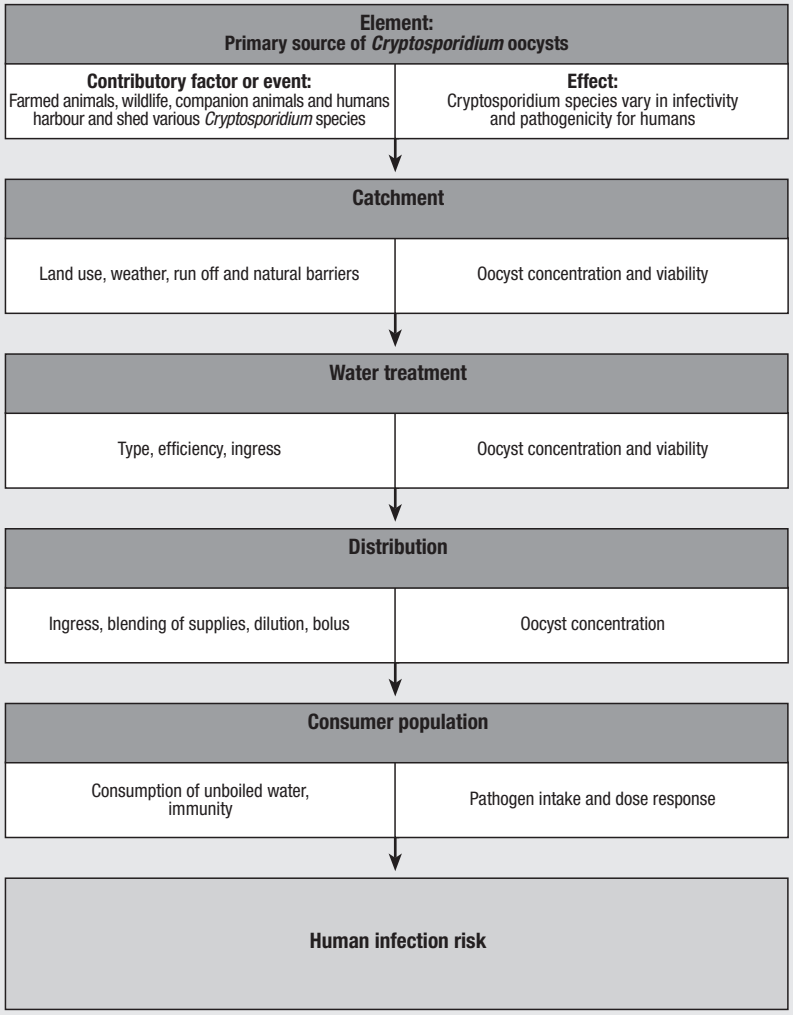

Fig. 1 | *Factors influencing drinking waterborne cryptosporidiosis: elements, contributory factors or events, and their effects.*

of regulatory change, and some of the recent developments in the detection and investigation of these outbreaks, especially the application of molecular typing assays.

IDENTIFICATION OF *CRYPTOSPORIDIUM* OUTBREAKS

Waterborne cryptosporidiosis outbreaks are identified through various surveillance systems, including passive and active morbidity reporting such as syndromic surveillance, laboratory reporting, sentinel surveillance systems, drug purchase or prescribing data, and media broadcasts. Poor water quality results, incidents or treatment failures will alert providers and authorities to a potential or existing outbreak. For example, following a water quality incident in the East Midlands, England in 2008 *(Table 3*; outbreak number 08/278), when oocysts were detected in treated water with a long history of non-detects in routine monitoring, syndromic surveillance data collected under the Qsurveillance scheme showed a significant increase in general practictioner consultations for diarrhoea and gastroenteritis in the week of the incident in the water distribution areas, compared with no increase in the unaffected areas [30]. Of the 33 cases of laboratory confirmed cryptosporidiosis identified during the outbreak investigation, 23 were *C. cuniculus*, the outbreak species (see below) [4] but QSurveillance data estimated an excess of 422 diarrhoea cases during the outbreak, an increase of about 25% over baseline weekly levels [30]. Thus, syndromic surveillance described the extent of cryptosporidiosis in the general population and provided reassurance that there was no further widespread impact.

The symptoms of cryptospordiosis are non-specific, so laboratory detection of the parasite, usually in stools, is required to confirm the infection. This is either by microscopy with prior staining using tinctorial, fluorescent or immunofluorescent stains, or immunoassay-based test kits, or more recently molecular assays. Routinely, detection in both clinical diagnostic and water testing laboratories, is of the genus: species identification can only

Table 3 | *Selected waterborne outbreaks of cryptosporidiosis in England and Wales 2001-2010 with molecular typing*

HPA Centre for Infections outbreak reference number	Government Office region	Year	Month	Setting or vehicle	Number of cases (laboratory confirmed)	Number of cases genotyped and result	Genotyping environmental isolates (where requested)	Strength of association (where indicated)
Drinking water								
02/1701	South East	2002-2003	November-January	Public water supply	➢ 31 (31)	28 *C. hominis*		Strong
05/552	South East	2005	September-November	Public water supply	140 (76)	76 *C. hominis*		Strong
05/790	Wales	2005-2006	October-January	Public water supply	231 (231)	225 *C. hominis* IbA10G2	Surface water and tap water also contained *C. hominis* IbA10G2 Other *Cryptosporidium* species and genotypes also detected in catchment samples	Strong
08/278	East Midlands	2008	June-July	Public water supply	➢ 400 (23)	23 *C. cuniculus* VaA18	Water in istribution, tap water and dead rabbit gut contents *C. cuniculus* VaA18	Strong
Recreational water								
01/347	South East	2001	June	School outdoor swimming pool	152* (10)	5 *C. hominis*		Possible
01/528	South West	2001	October-November	Club swimming pool	3 (3)	3 *C. hominis*		Possible
03/121	South East	2003	February	Swimming pool	20 (20)	4 *C. hominis* 11 *C. parvum*		Probable
03/220	Yorkshire and the Humber	2003	January-April	Swimming pool	66 (48)	21 *C. hominis*	Pool water and first backwash sample *C. hominis*, second backwash sample *C. parvum*	Strong
03/411	West Midlands	2003	August	Interactive water feature	122 (35)	31 *C. hominis* 1 C. *meleagridis*		Probable
03/409	South East	2003	August-September	Swimming pools	17 (17)	2 *C. hominis*		Strong
03/401	South West	2003	September	Interactive water feature at an open farm	63 (32)	29 *C. parvum* IIaA16G2 1 *C. parvum* IIaA19G2 2 *C. hominis*	Water feature *C. parvum* Goat and goat handler both *C. parvum* IIaA19G2;	Probable
~	North West	2004	March	Swimming pool	4 (4)	3 *C. hominis*		
04/186	Yorkshire and the Humber	2004	May-June	Swimming pool	7 (7)	3 *C. hominis*		
04/371	Yorkshire and the Humber	2004	October	Swimming pool	10 (9)	9 *C. hominis*		

Continues

Table 3 | *Continued*

HPA Centre for Infections outbreak reference number	Government Office region	Year	Month	Setting or vehicle	Number of cases (laboratory confirmed)	Number of cases genotyped and result	Genotyping environmental isolates (where requested)	Strength of association (where indicated)
05/554	South East	2005	September-October	Community swimming pools	➢ 88 (88)	76 *C. hominis* 7 *C. parvum*		Possible
05/623	London	2005	August-December	Swimming pools and community spread	➢ 129 (129)	13 *C. hominis*		Strong
06/36	North West	2006	January	Holiday park swimming pool	12 (11)	6 *C. hominis*		
~	West Midlands	2006	January	Club swimming pool	4 (4)	2 *C. hominis*		
06/481	North West	2006	June	Community swimming pool	6 (4)	4 *C. parvum*		
06/739	East Midlands	2006	June-July	Hotel swimming pool	13 (13)	7 *C. hominis*		
~	South East	2006	July	Comminity splash pool		2 *C. hominis*		
~	Wales	2006	September	Community swimming pool	9 (5)	4 C. hominis		
~	Wales	2006	October	Club swimming pool	13 (7)	7 *C. hominis*		
06/714	South West	2006	October	Hotel swimming pool	4 (4)	4 *C. hominis*		
06/607	Yorkshire and the Humber	2006	November	Club swimming pools	14 (14)	2 *C. hominis*		
06/668	East Midlands	2006	November	Holiday Park swimming pool	53 (27)	6 *C. hominis*		
06/670	North West	2006	November	Community swimming pool	4 (4)	2 *C. hominis* 2 *C. parvum*		
~	South East	2007	February	Community swimming pool	15 (5)	5 *C. hominis*		
~	West Midlands	2007	October	swimming pools	57 (39)	18 *C. hominis* 4 *C. parvum*		
08/375	Eastern	2008	November	School swimming pool	17 (17)	4 *C. hominis*		
09/64	Wales	2009	August	Community swimming leisure pool	106 (46)	44 *C. hominis*		
09/94	South West	2009	August	Caravan park swimming pool	7 (7)	7 *C. hominis*		
09/109	Yrokshire and the Humber	2009	September-October	Caravan park swimming pool	6 (5)	4 *C. hominis*		

Continues

Table 3 | *Continued*

HPA Centre for Infections outbreak reference number	Government Office region	Year	Month	Setting or vehicle	Number of cases (laboratory confirmed)	Number of cases genotyped and result	Genotyping environmental isolates (where requested)	Strength of association (where indicated)
~	Yorkshire and the Humber	2009	September-October	Swimming pool	6 (6)	1 *C. parvum*		
09/111	North West	2009	June-July	small warm swimming pool	8 (8)	3 *C. hominis*		
~	South East	2009	November	Community swimming pool	15 (11)	7 *C. hominis*		
~	Yorkshire and the Humber	2009	November	Community swimming pool	10 (?)	4 *C. hominis*		
~	North West	2010	September	Community swimming pool (swim club members)	48 (3)	3 *C. hominis*		
10/107	South East	2010	October	Community swimming pool	30 (19)	*C. hominis*		

**A concurrent norovirus outbreak accounts for some of the cases.*

be done by specific molecular "genotyping" assays, and is not usually performed by primary diagnostic laboratories. Not all diarrhoea patients will seek medical attention, have a stool sample taken or a *Cryptosporidium* test applied, and laboratory practice is varied. A request for "ova, cysts and parasites" will not include a test for *Cryptosporidium*: where not routinely sought, this must be specified on the request form. *Cryptosporidium* diagnosis is statutorily notifiable in only a few countries and cross-country comparisons are hampered by this [31]. For example, *Cryptosporidium* is included in Directive 2003/99/EC of the European Parliament and the Council of the European Union (EU), and cryptosporidiosis is therefore a notifiable disease within the EU with laboratory-confirmed case data collected through the European Surveillance System (TESSy). In 2009, reports were provided by 21 out of 31 EU and European Economic Area/European Free Trade Association countries; 8016 cases were reported across 13 countries and zero cases were reported by eight countries, an overall case rate of 2.7 per 100 000 population. The highest confirmed case rate was reported in Ireland (10.0 per 100 000 population) followed by the UK (9.3 per 100 000) and Belgium (4.1 per 100 000) [32]. Thus, *Cryptosporidium* is under diagnosed and underreported, but to varying extents. Even at a local level, testing and reporting practice is variable [33]. One study of IID in the UK has estimated the reporting ratio (*i.e.* the ratio of disease rates in the community and presenting to general practice relative to the rate of reported diagnoses to national surveillance) to be 8.2 (95% CI 2.1 to 31.7), estimating the annual number of cases in the community to be 43 834 (95% CI 11 393 to 168 655) [34]. This equates to an estimated annual incidence of 69.5 cases per 100 000 UK population, and the authors acknowledge the study may have underestimated *Cryptosporidium* rates as the case definition for acute gastroenteritis excluded cases of duration of illness over two weeks.

Surveillance data for England and Wales have shown that about 10% of reported cases of cryptosporidiosis are part of identified and reported outbreaks at a variety of settings *(Table 1)* [35], using the following definitions of an outbreak:

- an incident in which two or more people experiencing a similar illness are linked in time or place; or;
- a greater than expected rate of infection compared with the usual background rate for a place and time.

In investigations involving water, an incident may be defined as a suspected, anticipated or actual event involving microbial or chemical contamination of food or water. The Health Protection Agency (HPA) Centre for Infections (formerly the Communicable Disease Surveillance Centre) and local authorities in England and Wales have conducted structured surveillance of outbreaks of IID since 1992 [36]. Outbreaks are classified, according to the robustness of epidemiological and microbiological evidence, as definite, probable or possible defined from the following criteria:

a. the pathogen found in patients was also found in water samples;
b. documented water quality or treatment failure;
c. significant result from analytical epidemiologi-

cal study, demonstrating association between water and illness;

d. suggestive evidence that outbreak is water related from a descriptive epidemiological study, excluding obvious alternative explanations;

Which are combined to indicate the strength of association:

strong = a+c or a+d or b+c;

probable = b+d or a only or c only;

possible = b only or d only [37]. Outbreaks were summarised bi-annually until 2006 in the Communicable Disease Report, and are now reported in the Health Protection Report (www.hpa.org.uk).

A similar classification is in use in the USA where the Centers for Disease Control and Prevention (CDC), the Environmental Protection Agency (EPA), and the Council of State and Territorial Epidemiologists have collaborated on the Waterborne Disease and Outbreak Surveillance System (WBDOSS) for collecting and reporting data on waterborne disease outbreaks since 1971 for drinking water and 1978 for recreational water [28]. Two criteria must be met for a health event to be defined as a waterborne disease outbreak: 1) two or more persons must be linked epidemiologically by time, location of exposure to water, and illness characteristics, and 2) the epidemiologic evidence must implicate water or volatilization of water-associated compounds into the air surrounding the water as the probable source of illness. Outbreaks are classified according to the strength of both epidemiologic and clinical laboratory data, and environmental data implicating water as the vehicle of transmission:

Class 1 = epidemiologic and clinical laboratory data and environmental data are provided and are adequate;

Class 2 = epidemiologic and clinical laboratory data are provided and are adequate, but environmental data is not provided or are inadequate;

Class 3 = epidemiologic and clinical laboratory data are provided but are limited, and environmental data are provided and are adequate;

Class 4 = epidemiologic and clinical laboratory data are provided but are limited, and environmental data is not provided or are inadequate.

These classes, first delineated in the 1989-1990 surveillance report [38], have been updated to reflect the increasing use of molecular characterization of pathogens both in clinical specimens and environmental samples; molecular data that link people who had an identical water exposure are considered adequate to support a Class I or Class II assignment, and molecular data that link at least one person to the implicated water exposure now are considered adequate water quality data to support a Class I or Class III assignment [28]. The use of molecular data in the investigation of particular incidents and outbreaks is described below. However, as with many pathogens, the *Cryptosporidium* contamination event may have passed by the time of sampling and/or recognition of an outbreak. For *Cryptosporidium* the incubation period between ingestion and the onset of illness is long (up to two weeks) and the interpretation of microbial and water treatment process data requires careful interpretation. Differences in *Cryptosporidium* monitoring strategies for drinking water in the UK and USA are explored further below.

DRINKING WATERBORNE OUTBREAKS AS DRIVERS OF REGULATION

Although the first human cases of cryptosporidiosis were reported in 1976 [39, 40], the first outbreak linked to drinking water was identified in 1984, at Braun Station, Texas, USA [41]. Over 200 individuals were involved, when sewage contaminated a groundwater supply. The next outbreak was on an even larger scale: in 1987 an estimated 13 000 people were affected at Carrollton, Georgia, USA when mains water became contaminated from a surface supply during a period of operational irregularities at the conventional water treatment works [42]. In both cases the drinking water met existing water quality standards, based on WHO Guidelines for Drinking Water Quality, focussing on monitoring *E. Coli*. At the end of 1988 and into 1989, over 500 individuals were affected in Swindon and Oxford, England, when oocysts in contaminated surface source water broke through the conventional mains water treatment and in to supply [43]. These early outbreaks startled both public and water industry perception of drinking water in industrialised nations; filtration and chlorine disinfection were commonplace and assumed to control waterborne disease, and people no longer commonly became sick through the mains water supply. In 1989, *Cryptosporidium* was not even considered in the US Environmental Protection Agency (EPA) Surface Water Treatment Rule to control *Giardia* and viruses or in the companion Guidance Manual for Compliance with the Filtration and Disinfection Requirements for Public Water Systems, published in 1990. The 1993 outbreak in Milwaukee focussed the work initiated to understand the sources, routes of transmission, detection and prevention of spread of the parasite.

In addition to work seeking improvements in methods to detect, and water treatment to control *Cryptosporidium*, the outbreaks in the 1980's and early 1990's prompted regulatory agencies to develop rules for public health protection through monitoring, removal or inactivation. This has been approached differently in USA and UK.

In the USA, *Cryptosporidium* monitoring was first required under the Information Collection Rule (ICR) of 1997, requiring surface water sources of supplies to more than 100 000 people to be monitored for *Cryptosporidium* oocysts, *Giardia* cysts and viruses for 18 months. Sources with ≥ 1000 of either protozoan per 100 L, or viruses ≥ 100 per 100 L,

required final water monitoring. A total of 93% of 5829 surface water samples were reported as non-detects for *Cryptosporidium*. However, results based on testing small sub-samples and extrapolation of counts to the original volume are now considered unreliable. During the ICR period, a standard method was published by the EPA, stipulating the acceptable sampling filter types, elution and oocyst recovery processes and microscopical detection procedures for enumeration of oocysts, and, critically, that the oocyst count be expressed per volume of water sampled. In 1999 the method was validated for simultaneous detection of *Cryptosporidium* oocysts and *Giardia* cysts, currently published as EPA Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA, 2005. Method 1623 now supports promulgation of EPA's Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR), which is the current regulation for all public water systems that use surface water or ground water that is under the direct influence of surface water, finalised in 2006. This rule requires monitoring of source waters to determine the level of treatment required for *Cryptosporidium* reduction by removal or disinfection. Mean oocyst counts, based on a two year, monthly sampling programme, are used to classify ("bin") supplies in one of four categories and determine the extent of treatment required, if any, above conventional full treatment. Systems classified in higher bins must provide additional water treatment to further reduce *Cryptosporidium* levels by 90 to 99.7 percent (1.0 to 2.5-log), depending on the bin, using treatment and management options in a "microbial toolbox". All unfiltered water systems must provide at least 99 or 99.9 percent (2 or 3-log) inactivation of *Cryptosporidium*, depending on the results of their monitoring. Suitable removal is through filtration provided by granular media, cartridge filters or membranes, and approved disinfectants effective against *Cryptosporidium* are chlorine dioxide, UV, and ozone. Systems must conduct a second round of monitoring six years after completing the initial round to determine if source water conditions have changed significantly. The EPA estimates that full compliance with the LT2ESWTR will reduce the incidence of cryptosporidiosis by 89 000 to 1 459 000 cases per year, with an associated reduction of 20 to 314 premature deaths. Additional expected benefits include reduced exposure to other pathogens, such as *Giardia*, that can co-occur with *Cryptosporidium*.

In the UK, the Oxford and Swindon outbreak in 1987-1988 led to the establishment of the group of experts, chaired initially by Sir John Badenoch and latterly by Professor Ian Bouchier. A series of reports, published in 1990, 1995 and 1998 set out what was known about *Cryptosporidium*, its occurrence in the environment, its importance as a waterborne infection for humans, the outbreaks and lessons learned from them, the treatment requirements for *Cryptosporidium* deficiencies in detection and enumeration methods, the need for methods to establish oocyst viability, characterise the species present and their infectivity for humans, the risks from groundwaters infiltrated by surface waters, and established good epidemiological practice in investigation of outbreaks and the need for clarification in the role and function of incident and outbreak control teams and their members. A correlation between outbreaks and inadequacy occurring in elements of the multi-barrier approach (source, treatment, distribution, monitoring and response) was identified in the third report (available online at www.dwi.defra.gov.uk/research/bouchier/index.htm). This was initiated following an outbreak in north west London and Hertfordshire in 1997 when 345 cases were reported following contamination of a groundwater source, a type previously considered to present a low risk from *Cryptosporidium*, by infiltration of surface water containing oocysts [44].

Although the legal standards for all EU member states are set out in the European Drinking Water Directive 1998, and are themselves informed by the WHO guidelines on drinking water quality, national standards are also set. The first, and only, regulatory requirements for *Cryptosporidium* in finished water anywhere in the world were introduced in England and Wales in 1999 as part of the Water Supply (Water Quality) (Amendment) Regulations 1999 and incorporated in to the Water Supply (Water Quality) Regulations 2000 in England and 2001 in Wales. The key driver for these regulations was the lack of admissible evidence to prosecute the water company associated with an outbreak in 1995 in Torbay, south west England, when 575 cases occurred associated with a lowland river with direct abstraction and bankside infiltration of unfiltered water [45, 46]. The regulatory requirement mandated water undertakers to conduct risk assessments with respect to *Cryptosporidium* on all water treatment works, considering the source water, catchment characteristics and treatment provided. Sites with a "significant risk" classification, had to treat the water to ensure an average of less than 1 oocyst in 10 L of treated water supplied, measured by continuous sampling of at least 40 litres of water per hour. Compliance was demonstrated by continual monitoring and reporting of results to the regulator, the Drinking Water Inspectorate (DWI), unless all particles > 1μm were continuously removed. The initial risk assessment reported in the DWI's annual report in 2001 (http://dwi.defra.gov.uk) identified 332 sites as being at significant risk, of which 158 were works treating surface waters and 174 were groundwater abstractions. Some sites were decomissioned and others subjected to improvement. In 2004, the DWI reported that none of the 146 307 samples taken between 2000 and 2003 exceeded the treatment standards, with most samples below 0·02 oocysts/10 L.

Improvements in drinking water quality driven in part by the England and Wales regulations,

and by long-term water company investment programmes, led to a substantial reduction in both *Cryptosporidium* cases, especially in the first half of the year, and in reported mains drinking water outbreaks [18, 47] at a time when swimming pool outbreaks appeared to increase in number (see below). Only four outbreaks linked to public water supplies have been recorded in England and Wales since 2000; one in 2002, two in 2005 and one in 2008, all with strong evidence for association with drinking water (*Table 3*). In November and December 2002 an outbreak involving 31 laboratory confirmed cases caused by *C. hominis* was reported in a population of 158 558 in South East England served by a mixture of water from a groundwater source and a surface water-treatment plant at significant risk, and where the continuous monitoring samples never exceeded treatment standards (*Table 3*, outbreak 02/1701) [48]. A second outbreak of 140 laboratory confirmed cases caused by *C. hominis* in the same area occurred between September and November, 2005 and once again, oocyst counts were below the treatment standard (*Table 3*, outbreak 05/552). The recognition that even small numbers of oocysts detected in drinking water can cause outbreaks contributed to the revocation of the treatment standard in the amended water quality regulations, The Water Supply (Water Quality) Regulations 2000 (Amendment) Regulations 2007. Furthermore, an outbreak in north west Wales in 2005, involving 218 cases of cryptosporidiosis (again, *C. hominis*) was linked to a surface water supply derived from a sparsely populated catchment. The treatment works, despite the absence of effective treatment or barriers in the catchment to remove *Cryptosporidium*, was not continually monitored as it had not been given a significant risk assessment [49]. The outbreak was controlled in the short term by a notice to boil drinking water, which was in place for 9 weeks until a UV treatment plant could be installed, although oocyst disinfection (inactivation) was not permitted by the regulations at the time, which focussed on oocyst removal. The elimination of the treatment standard in the 2007 regulations permitted application of disinfection such as UV for the control of *Cryptosporidium* in water supplies. Never-the-less, drinking water remains a risk factor for cryptosporidiosis in England and Wales [18].

As demonstrated above, outbreaks of cryptosporidiosis have been reported through drinking water that met WHO guideline microbiological standards and/or the *Cryptosporidium* treatment standard imposed in England and Wales. Subsequently, a preventive, risk based approach, derived from the food industry [50], in the form of the requirement for a water safety plan [51], now complements microbiological guidelines, and is therefore incorporated in further amendments to the regulations in 2010 in England and Wales as comprehensive risk assessments. A water safety plan is a systematic inventory of all hazards (including *Cryptosporidium*), an evaluation of the significance of these hazards and of the efficacy of control measures taken, and spans source water catchment, treatment and distribution of water supplies. The risk assessment is supported by testing and enforcement. However, one of the key differences between the USA and UK approaches remains: the former is historically based on source water monitoring for *Cryptosporidium* to inform subsequent levels of treatment required and the latter has the historical legacy of an emphasis on final water monitoring. Regulations supporting the Drinking Water Directive and water safety plan approach require raw water monitoring to identify risks to deterioration of raw water quality; there is no list of parameters for this purpose as it is up to the water company to assess the risks and monitor and treat accordingly.

SWIMMING POOL OUTBREAKS AND LACK OF REGULATION

Although the WHO *Guidelines for safe recreational waters* [52] provide a basis for standards, in swimming pool settings, in contrast with drinking water, there is a lack of legislation. The WHO guidelines, which are currently under revision, provide an authoritative referenced review and assessment of the health hazards associated with swimming pools, their monitoring and assessment, and activities available for their control through education of users, good design, construction, operation and management, and address a wide range of types of hazard, including water quality, physical hazards (leading to drowning and injury), contamination of associated facilities and air quality. In the USA, state and local governments establish and enforce regulations for protecting recreational water from contaminants but no federal agency has authority over treated recreational water and, apart from legislation to prevent entrapment, no minimum design, construction, operation, disinfection, or filtration standards exist. Swimming pool codes are enforced by individual state and local public health agencies but there is variation in regulation, compliance and enforcement. In the EU, the Bathing Water Directive 2006 sets out quality standards for natural waters designated for bathing but this does not cover treated waters. In the UK, the publication *Swimming pool water. Treatment and quality standards for pools and spas* [53] provides authoritative guidance and is viewed as best practice. Therefore, in a court of law, swimming pool operators would be, and indeed have been, prosecuted under the Health and Safety at Work etc. Act 1974, and the Management of Health and Safety at Work Regulations 1999, for causing an outbreak by failing to follow this guidance [54].

Globally, recreational waterborne outbreaks of cryptosporidiosis are reported slightly more commonly than drinking waterborne outbreaks: in the seven years between January 2004 and December 2010,

out of 120 reported waterborne *Cryptosporidium* outbreaks, 46% were associated with drinking water and 54% with recreational waters [23]. However, this analysis belies an apparent increase in swimming pool related *Cryptosporidium* outbreaks seen, for example, in the UK and USA. Between 1992, when surveillance for waterborne IID outbreaks began [36] and 2011, there were 56 outbreaks linked to swimming pools and 32 linked to public water supplies. However, just 29 (34%) of the swimming pool outbreaks were reported in the first ten years (1992 to 2001) compared with 56 (66%) in the following ten years (2012 to 2011) (Gordon Nichols, personal communication of provisional data). In the USA, *Cryptosporidium* outbreaks at recreational water venues also increased from 29 in 2005-6 to 60 in 2007-8 [28]. While the emergence of *Cryptosporidium* may be a continuing factor, other contributing factors may include changes in detection, investigation, and reporting of waterborne disease outbreaks driven by improved resources for the WBDOSS and strengthening of the outbreak response [28]. It is likely that recently improved understanding of risks associated with swimming pools and their investigation, has contributed to the apparent rise in outbreaks at these settings.

APPLICATION OF MOLECULAR ASSAYS FOR SPECIES DETERMINATION AND INVESTIGATION OF THE PROPAGATION OF OUTBREAKS

Molecular detection offers some advantages over standard methods such as EPA Method 1623, which are based on oocyst counts by immunofluorescence microscopy, such as detection of small numbers of organisms and potential for genotyping leading to species determination, this has not yet been adopted for operational or regulatory monitoring of drinking water. This is because only those oocysts containing sporozoites, and thus DNA, will be detected and so far, no reliably quantitative molecular method has been validated to replace oocyst counts. However, without assays determining the species/genotype, viability or infectivity, all oocysts detected by microscopy must be assumed to present a public health risk *(Figure 1)*. Viability and infectivity assays have been reviewed recently by Kothavade, who highlights the difficulties in applying these to the small numbers of oocysts often present in water samples, lack of interlaboratory trials and validation and difficulties in interpretation of the data [55]. In contrast, some genotyping assays have been standardised and applied to counted oocysts from microscope slides; thus both sets of data are collected: the oocyst count and the species, improving the data for assessment of risk to public health.

Cryptosporidium genotyping may be undertaken using material fixed and stained on microscope slides generated during *Cryptosporidium* monitoring by extracting *Cryptosporidium* DNA and applying conventional polymerase chain reaction (PCR) of the small subunit ribosomal RNA (ssu rRNA) gene prior to sequencing of amplicons (the benchmark method) or, more recently, using real-time PCR platforms *(Figure 2)*. Other methods, such as laser capture microscopy and reverse line hybridization are at developmental stages, and loop-mediated isothermal amplification have yet to be validated independently [55]. The benchmark assay is a specialist test requiring equipment and skill outwith the scope of most routine diagnostic or detection laboratories. The large genetic diversity in *Cryptosporidium* ssu rRNA sequences from source waters makes analysis complex. For example, an up to date reference database to compare accurate DNA sequences and subsequent phylogenetic analysis with proper interpretation are required, coupled with up to date knowledge of host-parasite relationships [56]. Rueker and colleagues have provided a detailed description and discussion of the sequence and phylogenetic analysis process, especially important in the identification of environmental *Cryptosporidium* isolates [56], providing a benchmark process. An ongoing Water Research Foundation (WaterRF) programme aims to further refine the benchmark assays and develop and validate simplified, rapid, assays for routine use in water testing laboratories for the differentiation of human pathogenic species (for this purpose defined as *C. hominis*, *C. parvum* and *C. meleagridis*) from animal-infective species (WaterRF 4284) (http://waterrf.org) *(Figure 2)*. Although the simplified assay, as proposed, is not likely to differentiate *C. cuniculus*, this species will be included in the *C. hominis* detections and may be differentiated by sequencing of PCR products, which is required for definitive results *(Figure 2)*.

Although there is no standard method for subtyping *Cryptosporidium* species, sequence analysis of the *gp60* gene is informative for *C. parvum* and *C. hominis* to a certain extent and sequence data can be compared readily [57]. Although the epidemiology of *gp60* subtypes was reviewed by Xiao in 2010 [57], new variants are frequently identified. The utility of the analysis has been demonstrated during the investigation of both zoonotic [58] and anthroponotic transmission, as illustrated in the investigation of waterborne outbreaks described below. However, single locus typing will under-estimate diversity, and a standardised, validated, internationally accepted, multi-locus scheme is required for epidemiological investigations of each species [59]. How subtype variation relates to virulence and pathogenicity is unclear; in fact, only putative virulence factors have been identified so far, although *gp60* subtypes have been associated with varying severity of illness [60]. Despite this, genotyping has proved to be beneficial to the epidemiological investigation of cryptosporidiosis, and extracted DNA can be stored long-term and re-tested retrospectively.

In non-outbreak situations, the data from typing sporadic cases of cryptosporidiosis provides a

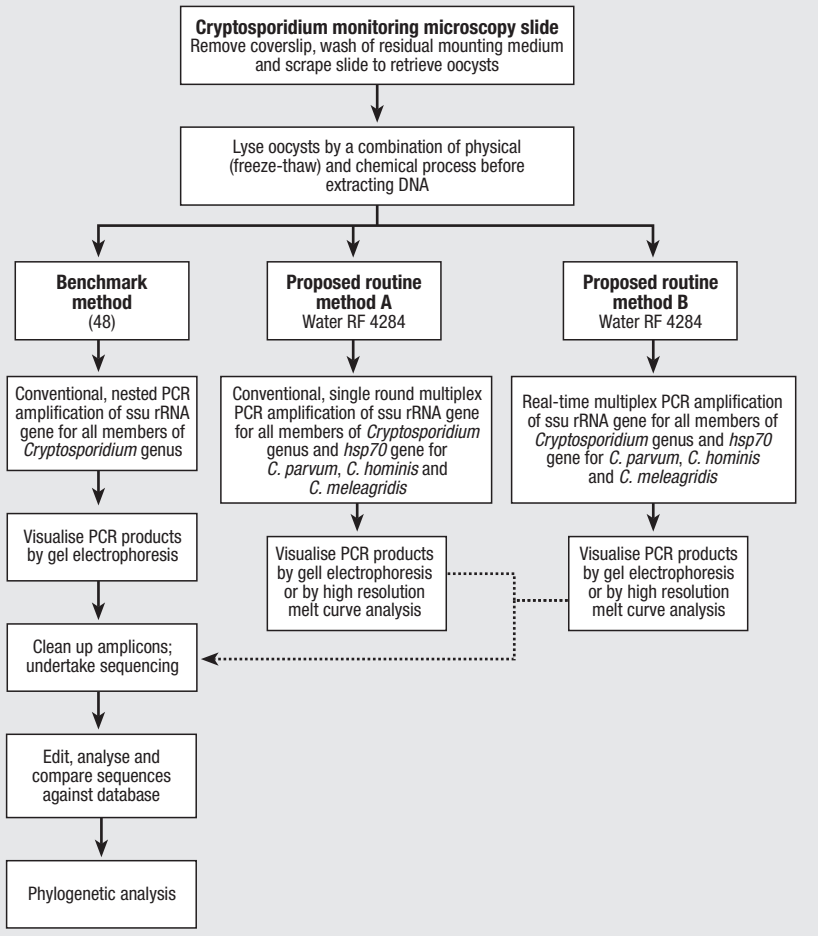

Fig. 2 | *Workflows for genotyping* Cryptosporidium *from regulatory monitoring microscopy slides.*

baseline against which trends can be identified and changes monitored. Since the improvements in water quality influenced by the 2000/1 regulations in England and Wales, the spring peak identified as being caused by *C. parvum* declined substantially [61]. Depite this, drinking water remains a risk for illness caused by *C. parvum*, as identified by Lake and colleagues in a case-control study design to investigate wider environmental and socioeconomic risk factors for human cryptosporidiosis in England and Wales [62]. In contrast to the decline in *C. parvum* cases in the spring, there has been no reduction in late summer/early autumn cases of *C. hominis* which predominates at this time of year. This is especially obvious in 2012, and recreational waters are implicated [63]. More swimming pool related outbreaks are caused by *C. hominis* than *C. parvum*, although both have occurred in the same outbreak *(Table 3)*, and may reflect multiple episodes of contamination.

When applied to *Cryptosporidium* isolates from catchment studies or routine drinking water monitoring, genotyping data can also further refine human health risk assessment by differentiating human pathogenic from solely animal-associated species and supports the other information gathered under drinking water regulatory frameworks. For example, a one year survey of *Cryptosporidium* oocysts detected in the Scottish Water Routine *Cryptosporidium* Monitoring Programme identified the species or genotypes present in 62.5% of 1042 oocyst-positive slides [64]. A high diversity of *Cryptosporidium* species and genotypes was present in source (no. = 456) and treated drinking (586) waters, with 2 or more in 16.9% samples; human-pathogenic species were present in fewer samples than non-pathogenic species. In source waters, *C. andersoni* (which is host-adapted to cattle) was most frequently identified (25.2% samples) followed by *C. parvum* (11.2%) and *C. ubiquitum* (7.2%). In drinking waters, *C. ubiquitum* was most frequent (12.6% samples), followed by *C. parvum* (4.3%) and *C. andersoni* (4.1%). In a long-term study of source waters in the agriculturally intensive South Nation River catchment in Ontario, Canada, *Cryptosporidium* species associated with livestock made up 39% of the total molecular detections, compared with wildlife associated species and genotypes accounted for 55% and *C. hominis* and *C. parvum* 1.6%, indicating a small risk to hu-

mans [56]. A study in Australia, where recreational activities within 2 km of drinking water sources is prohibited, compared *Cryptosporidium* species in recreational water and non-recreational water catchments [65]. This revealed a predominance of *C. hominis* in recreational water catchments which allowed swimming and camping, compared to non-recreational catchments which had a lower prevalence of *Cryptosporidium* and all the samples genotyped were *C. parvum*. Increasing population was strongly correlated with an increase in the prevalence of *Cryptosporidium* recreational catchments, and the data support government policy limiting activities to the outer catchment [65]. The predominance of host-derived species in some catchments, contributes to effective evaluation and selection of management practices to reduce *Cryptosporidium* contamination in source waters.

Retrospective analysis of clinical isolates can be enlightening, identifying, for example, that the Milwaukee outbreak was caused by *C. hominis* [66], where previous uncertainty over the origin of the oocysts identified cattle, slaughterhouses, and human sewage as potential candidates [26]. Further analysis of isolates collected in the Torbay outbreak [46] and north west London and Hertfordshire outbreak [44] established that these were also caused by *C. hominis* [67]. Even where species identification has been undertaken, further analysis of isolates from cases may be required to investigate outbreaks. Retrospective analysis of *C. parvum* isolates by multilocus fragment typing enabled more accurate mapping of outbreak-related cases to water supply zones [68]. Some nine years after the Milwaukee outbreak, *C. hominis* isolates, further analysed for variation in the *gp60* gene, were identified as indistinguishable from the outbreak isolate, year round in Milwaukee wastewater indicating continuing, stable transmission of human cryptosporidiosis in the city [69]. Analysis of *C. parvum* isolates from patients linked to a swimming pool outbreak in Stockholm, Sweden in 2002, at three loci (*gp60*, TP14 and *hsp70*) identified that two separate outbreaks had occurred simultaneously, as the genotypes segregated people using an outdoor pool from those that swam only in the indoor pool [70].

Latterly, genotyping methods have been applied in "real time" during incident and outbreak investigations and have been demonstrated to assist in attributing sources and establishing correct interventions. In the UK, isolates from clinical cases are genotyped routinely to the species-level, and in some outbreaks additionally from suspected sources *(Table 3)*. For example, during investigation of a drinking waterborne outbreak caused by *C. hominis* in north west Wales in 2005 (outbreak number 05/790), isolates found in clinical cases were indistinguishable by sequence analysis of the ssu rRNA gene and the *gp60* gene from isolates in the surface water source and in the treated water supply to the affected area, adding strength to the epidemiological evidence for the association with drinking water [49,71]. Although a diversity of *Cryptosporidium* species was identified in the catchment, the outbreak *C. hominis* subtype, IbA10G2, was only found in source waters under the influence of sewage contamination, and in the tap water, highlighting the need for water companies to throughly document wastewater inputs in surface waters [72]. During a water quality incident in the East Midlands, England in 2008 (*Table 3*, outbreak number 08/278), isolates in treated water were identified as *C. cuniculus* before any human illness was identified, and were matched subsequently by sequencing the ssu rRNA, *hsp70* and *gp60* genes to those from a dead rabbit found in a chlorine contact tank and 23 ensuing human cases [4]. This outbreak established *C. cuniculus* as a human pathogen. In two out of three drinking waterborne outbreaks in Northern Ireland in a single year, the confirmation of *C. hominis* re-directed the investigations away from farmed animals and towards raw sewage contamination in one outbreak and wastewater in the other, illustrating how genotyping isolates from cases can also eliminate alternative suspected sources [73].

Secondary spread from initial outbreaks at swimming pools, for example, through other swimming pools and community settings such as nurseries can lead to community wide outbreaks, as has been shown in Japan [74] and in 2007 in Utah, USA [75]. The ability of *Cryptosporidium* to survive for over 10 days even in properly chlorinated water, the protracted incubation period (commonly 5 to 7 days) and possible diagnostic delay which prolongs the time between infection and epidemiologic link with the source of the outbreak contribute to this, as does behaviour of cases who continue to swim while ill. They can introduce the protozoan to other recreational water venues, as was shown in the Utah outbreak where an estimated 20% of cases swam while ill with diarrhoea and identified approximately 450 potentially contaminated recreational water venues [75]. Subsequent to the Utah outbreak, increases in cases in 2007 were seen in neighbouring states of Colorado, Idaho, New Mexico, and Iowa. Subtyping 57 *C. hominis* isolates at the *gp60* gene identified the same, previously rare subtype (IaA28R4) in 40 (70%) samples [76]. Unfortunately, none of the Utah samples were available for typing, and it is impossible to know whether this increase was related. This would have been a powerful investigation in to the spread of cryptosporidiosis and highlights the importance of the availability of isolates for further investigation.

Thus, the benefits of genotyping in outbreaks are:

- characterising the outbreak in terms of the cause of the cryptosporidiosis;
- linking cases with each other and monitoring their spread, or excluding cases with non-related isolates;

- linking cases with isolates in suspected sources, or excluding other sources;
- providing supporting data for further investigation of the source of contamination;
- providing additional evidence for the strength of evidence for the association with water;
- providing new data for prevention and control of further outbreaks.

However, there are additional challenges to genotyping most especially from environmental samples, requiring mitigation strategies:

- sensitivity, as small numbers of oocysts may be present on slides and in the pesence of potential PCR inhibitors;
- specificity, as there is difficulty in selecting target gene sequences that are both conserved within all *Cryptosporidium* species but different or absent in other genera;
- not all isolates from environmental samples are typable, even when present in large numbers, for unknown reasons;
- in concurrent *Cryptosporidium* populations, it may be difficult to identify multiple sequences present, especially of minor genotypes which may be underdetected;
- genotypes have been found in source waters which have no known host; there is a need for more information on *Cryptosporidium* shedding by most host species;
- a limited amount of *Cryptosporidium* DNA may be available for subtyping from environmental samples;
- assays are time consuming, specialised and costly.

CONCLUSIONS

Despite the knowledge gained since the first drinking waterborne outbreaks, there are still gaps that need to be filled. Waterborne outbreaks have occurred where small numbers of oocysts have been detected, and conversely, high numbers of oocysts do not necessarily lead to increased disease [76]. This reflects the multifactorial dynamics of waterborne disease, including human behaviour (water consumption) and immunity of the exposed population, as well as the potential infectivity of the oocysts for humans, determined by their species and viability *(Figure 1)*. These present analytical and interpretive challenges to public health and quantitative microbial risk assessment. Nevertheless, early detection, investigation and appropriate control of outbreaks can reduce their impact, and are facilitated by molecular methods to establish the relationship between isolates from cases and suspected sources.

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Conflict of interest statement

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The importance of waterborne disease outbreak surveillance in the United States

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Abstract. Analyses of the causes of disease outbreaks associated with contaminated drinking water in the United States have helped inform prevention efforts at the national, state, and local levels. This article describes the changing nature of disease outbreaks in public water systems during 1971-2008 and discusses the importance of a collaborative waterborne outbreak surveillance system established in 1971. Increasing reports of outbreaks throughout the early 1980s emphasized that microbial contaminants remained a health-risk challenge for suppliers of drinking water. Outbreak investigations identified the responsible etiologic agents and deficiencies in the treatment and distribution of drinking water, especially the high risk associated with unfiltered surface water systems. Surveillance information was important in establishing an effective research program that guided government regulations and industry actions to improve drinking water quality. Recent surveillance statistics suggest that prevention efforts based on these research findings have been effective in reducing outbreak risks especially for surface water systems.

Key words: drinking waters, epidemiology, surveillance systems.

Riassunto *(L'importanza della sorveglianza delle epidemie delle patologie trasmesse con le acque negli Stati Uniti)*. L'analisi delle cause delle epidemie di malattie associate con l'acqua potabile contaminata negli Stati Uniti ha contribuito ad indirizzare le misure di prevenzione a livello nazionale, di stato e locale. Questo articolo descrive i cambiamenti nella natura delle epidemie di malattie nei sistemi idrici pubblici fra il 1971 e il 2008 e discute l'importanza del sistema di sorveglianza delle epidemie associate all'acqua stabilito nel 1971, in un contesto di collaborazione. Un crescente numero di notifiche sulle epidemie attraverso i primi anni 80 aveva sottolineato l'importanza dei microrganismi patogeni nelle acque potabili. Le indagini sulle epidemie hanno permesso di identificare gli agenti eziologici responsabili e le deficienze nei trattamenti e nella distribuzione delle acque potabili, specialmente il rischio elevato associato con sistemi che utilizzavano acque superficiali non filtrate. Le informazioni derivanti dalla sorveglianza sono state importanti per stabilire programmi efficaci di ricerca per indirizzare i regolamenti governativi e le azioni industriali allo scopo di migliorare la qualità delle acque potabili. Statistiche recenti sui dati della sorveglianza suggeriscono che le azioni di prevenzione basate sui risultati di queste ricerche sono state efficaci nel ridurre i rischi di epidemie nei sistemi idrici pubblici.

Parole chiave: acque potabili, epidemiologia, sistemi di sorveglianza.

INTRODUCTION

Statistics on disease outbreaks associated with contaminated drinking water in the United States have been collected and analyzed for various time periods since 1920 [1-6]. These analyses have informed research, regulatory, and industry actions to improve drinking water quality and reduce waterborne disease risks. For example, the causes of outbreaks reported during 1961-1970 [4] was deliberated during Congressional committee hearings [7], contributing to the passage of the Safe Drinking Water Act [8, 9]. This [4] also led to a partnership between the US Environmental Protection Agency (EPA) and Centers for Disease Control and Prevention (CDC) to improve the detection, investigation, and reporting of waterborne outbreaks. The partnership has continued since 1971 with the periodic publication of outbreak statistics associated with drinking and recreational water, the most recent of which are for 2007-2008 [10, 11]. In this article, the Author focuses on the changing causes of outbreaks in public water systems during the past 38 years to illustrate the importance of waterborne disease surveillance.

WATERBORNE DISEASE OUTBREAK SURVEILLANCE

Public health agencies or health departments in the states, US territories, and localities have primary responsibility for detecting and investigating water-

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borne outbreaks, and during an outbreak investigation, they can request epidemiologic, environmental health, engineering, and laboratory assistance from the EPA and CDC. As part of the surveillance system, outbreak information is voluntarily reported to the CDC. This information includes characteristics of the outbreaks (*e.g.*, etiology, number of cases, illness severity, location), epidemiologic data (*e.g.*, attack rates, vehicle-specific risks), and results from environmental and engineering investigations (*e.g.*, water quality data, contamination sources, adequacy of disinfection and other water treatment deficiencies).

The outbreak reports are evaluated by EPA and CDC personnel to determine whether the epidemiologic evidence is sufficient to implicate water as the probable source of illness. Outbreaks with limited environmental data are included in the surveillance database, but outbreaks that lack necessary epidemiologic data are not. Single cases of chemical or toxin poisonings may be included if drinking water exposure is well documented. Additional information about the surveillance system and criteria for evaluating outbreak reports is available in the most recently published surveillance summary [10].

The surveillance system contains information for 953 outbreaks associated with contaminated drinking water during 1971-2008; 88% (no. = 834) were associated with water intended for drinking. The remaining outbreaks were associated with water not intended for drinking (no. = 81) and water of unknown intent (no. = 38). Legionellosis outbreaks associated with contaminated drinking water were not systematically included in the surveillance system until 2001; however, outbreaks that occurred before 2001 were recently added to the database after a thorough search of CDC and state health department records [10].

This article considers only those outbreaks reported in public water systems. Of the 834 outbreaks associated with water intended for drinking, 733 (88%) were reported in public water systems; 12% were associated with individual water systems (no. = 88) and bottled or bulk water (no. = 13). A public system is defined as either a community or non-community system that provides drinking water through a distribution system with at least 15 service connections or that regularly serves at least 25 persons [12]. Community systems serve the same persons year round; non-community water systems serve the public but do not serve the same persons year round. Of the 152 979 public water systems in the United States, 34% are community systems and 66% are non-community systems [12]. Community systems serve 77% of the US population with 70% of this population supplied by systems that use surface water [12].

DRINKING WATER REGULATIONS

The Safe Drinking Water Act of 1974 and its subsequent 1986 and 1996 amendments authorized the EPA to set national standards to protect public drinking water and its sources against naturally occurring or man-made contaminants [9, 13, 14]. These standards include health-based maximum levels for microbiologic, chemical, and other contaminants in drinking water and water treatment performance criteria for their removal or inactivation. The EPA regulations apply only to public water systems. To help interpret the outbreak statistics reported in this article, it is necessary to be familiar with the various regulations and when they were promulgated *(Table 1)*. Regulations that protect against waterborne exposure to pathogens include the Surface Water Treatment Rule and its amendments [15-21], the Total Coliform Rule [22-25], and the Ground Water Rule [26, 27]. In addition, the EPA periodically publishes a list of contaminants that will be evaluated for potential regulatory action; *Legionella pneumophila* was included in the 2009 contaminant list [28, 29].

The Surface Water Treatment Rule and its amendments specify water-treatment techniques (*e.g.*, filtration and disinfection), monitoring, and performance criteria for systems that use surface water sources or ground water sources that are under the direct influence of surface water. The 1989 rule required these systems to reduce the source water concentration of *Giardia* cysts and viruses by at least 99.9% and 99.99%, respectively [15]. In addition, a detectable disinfectant residual must be maintained throughout the entire distribution system, and filtration performance must be assured by monitoring turbidity levels. The 1998 rule specified 99% removal of *Cryptosporidium* oocysts for filtered systems that serve 10 000 or more people [16]. It also required more stringent turbidity criteria and the monitoring of individual filters.

Table 1 | *National regulatory requirements for microbial contaminants*

Regulation	Date promulgated	Compliance date
Total Coliform Rule	June 1989	December 1990
Surface Water Treatment Rule	June 1989	December 1990
Interim Enhanced Surface Water Treatment Rule	December 1998	January 2002
Filter Backwash Recycling Rule	June 2001	June 2004-2006
Long Term 1 Enhanced Surface Water Treatment Rule	January 2002	January 2003
Long Term 2 Enhanced Surface Water Treatment Rule	January 2006	January 2009-2011
Ground Water Rule	January 2007	December 2009

The 2002 rule extended *Cryptosporidium* removal requirements to systems serving fewer than 10 000 persons, updated watershed control requirements to include *Cryptosporidium* as a concern for unfiltered systems, and addressed risk trade-offs with disinfection byproducts [17]. The 2006 rule required additional treatment for all unfiltered surface water systems and for filtered systems with significant levels of *Cryptosporidium* in their source waters [18-20]. Treatment requirements are based on levels detected during monitoring. Systems serving at least 100 000 people began monitoring in October 2006, and systems serving fewer than 10 000 people began monitoring in October 2008. Compliance is required within three years after monitoring results were available, and systems are required to conduct additional monitoring six years after the initial monitoring to determine if source water conditions have changed significantly. The Filter Backwash Recycling Rule [21] helps ensure that recycle practices do not compromise the ability of treatment plants to remove pathogens, especially *Cryptosporidium*.

Coliforms in drinking water may indicate problems with water treatment (*e.g.*, the system's treatment is inadequate to deal with source water contamination or is not performing properly) or problems in the distribution system. The Total Coliform Rule requires public water systems to monitor for indicators of fecal contamination and when coliforms are found, take corrective action and report violations to the state regulatory agency and the public [22, 23]. Corrective actions include upgrades to water treatment or the distribution system and source water protection programs to prevent contamination. In 2007, EPA established an advisory committee to evaluate health risks associated with the degradation of water quality in the water distribution system and to suggest improvements to that rule [24]. Revisions to the rule were proposed in 2010 [25] and addressed these issues: cross-connections and backflow, mains repair and construction, storage facilities, pressure and intrusion, biofilm, nitrification, and contaminant accumulation.

The Ground Water Rule provides for increased protection against bacteria and viruses for systems that use ground water sources or that mix surface and ground water if the ground water is added to the distribution system without treatment [26, 27]. Periodic sanitary surveys are required to identify wells and springs that are susceptible to fecal contamination and to evaluate treatment technologies, operation, and management. Compliance monitoring is required to ensure that treatment reliably achieves 99.99 percent inactivation or removal of viruses.

OUTBREAKS REPORTED IN PUBLIC WATER SYSTEMS

The 733 outbreaks reported in public water systems resulted in 579 582 cases of illness and 116 deaths *(Table 2)*. Included among the 359 community system outbreaks is a single outbreak of 20 cases associated with both community and individual water systems. Included among the 353 non-community system outbreaks is a single outbreak of 1450 cases associated with non-community and individual water systems. In these two outbreaks, wide spread contamination of ground water affected both public system and individual wells. For 21 public system outbreaks, insufficient information was available to classify the system as either community or non-community. Nineteen of the outbreaks with insufficient information were legionellosis outbreaks that occurred before 2001 and had just recently been added to the surveillance system; the other two outbreaks were associated with contamination at the point of water use.

Table 2 | *Waterborne disease outbreaks and illness severity, public systems, 1971-2008*

Water system type	Number of outbreaks	Cases of illness	Deaths
Community systems*	359	521 617	83
Non community systems	353	57 641	4
Unknown system type	21	324	29
Total: public water systems	733	579 582	116

**403 000 illnesses and 50 deaths in one outbreak in Milwaukee 1993.*

Although community and non-community water systems reported a similar number of outbreaks, more illnesses occurred in community system outbreaks. Excluding the 403 000 illnesses in the 1993 Milwaukee cryptosporidiosis outbreak [30, 31], outbreaks in community systems had twice the number of illnesses than outbreaks in non-community systems. The annual number of outbreaks in public water systems decreased throughout the 38-year period *(Figure 1)* as did their magnitude *(Table 3)*. From a peak of 45 outbreaks in 1981, only 6 outbreaks were reported in 1998. Since 2001, annual reports ranged from 7 to 17 outbreaks. In recent years, outbreaks occurred primarily in small water systems with fewer cases of illness being reported. The median number of illnesses reported in public water system outbreaks during 2001-2008 was one-fourth that reported 1971-1980.

Outbreak etiologies

An etiology was identified for 414 (56%) of the 733 outbreaks reported during 1971-2008 *(Figure 2)*. Pathogens were identified in 341 (46%) outbreaks; parasites and non-Legionella bacteria were the most frequently identified pathogens. Chemicals caused acute illness in 73 (10%) outbreaks. Most frequently identified were high levels of copper associated with corrosive water and the overfeeding of fluoride or sodium hydroxide during water treatment. These three chemicals caused 26 (36%), 12 (16%), and 7 (10%) of the chemical outbreaks, respectively. Other chemical contaminants included nitrate, herbicides, pesticides, and various types of oil products. An etiology was not determined in 319 (44%) outbreaks

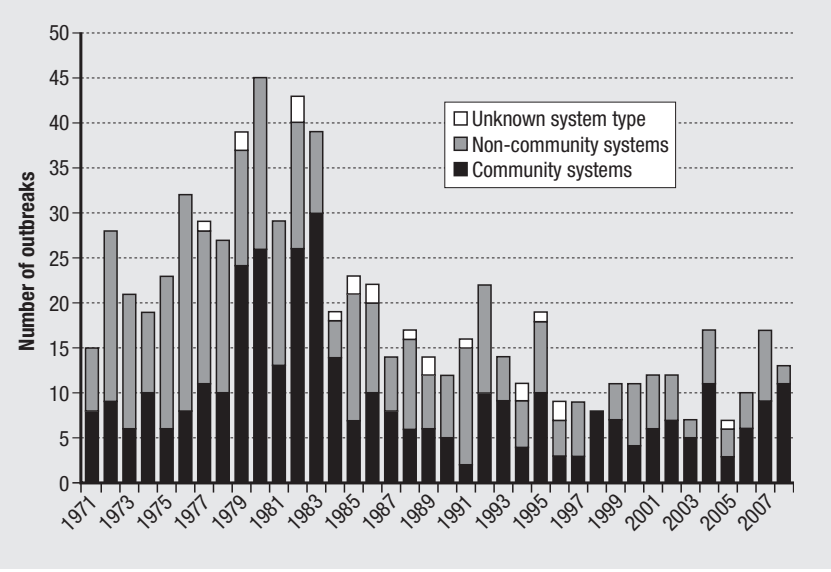

Fig. 1 | *Number of waterborne disease outbreaks reported annually, public water systems, 1971-2008 (no. = 733).*

either because timely specimens were not collected and analyzed or because laboratory analyses could not identify an agent. In 317 of these outbreaks, 82 869 cases of acute gastroenteritis were reported; two outbreaks resulted in 94 cases of severe watery diarrhea that lasted for months. The outbreaks of chronic gastroenteritis occurred in Illinois [32] in 1987 and in Oklahoma [33] in 1988.

Parasites

Giardia intestinalis was identified as the sole pathogen in 115 (87%) of the parasitic outbreaks and was responsible for 28,161 cases. *Cryptosporidium* spp. were identified in 12 (9%) parasitic outbreaks and caused 421 301 cases; 403 000 of these cases were attributed to *C. hominis* in the Milwaukee outbreak. Two outbreaks and 63 cases were attributed to *Entamoeba histolytica*, and two outbreaks and 103 cases were attributed to *Cyclospora*. An outbreak of *C. cayetanensis*, a rarely reported source of drinking water outbreaks in developed countries, occurred in Puerto Rico in 2008 [10]; a previous cyclosporiasis outbreak occurred in Chicago in 1990 [34]. The first association of a laboratory-confirmed primary amebic meningoencephalitis with a drinking water system occurred in 2002; two previously healthy children died in Arizona [35]. The children lived in a neighborhood that received water from untreated wells. *Naegleria fowleri* was found in water from a storage tank connected to one of the wells, and the water tested positive for coliforms. In the remaining two parasitic outbreaks, *G. intestinalis* and *Cryptosporidium parvum* were identified in one, and *G. intestinalis* and *E. histolytica* were identified in the other.

Table 3 | *Trends in magnitude of waterborne disease outbreaks, public systems, 1971-2008*

Time period	Cases of illnesses		
	Largest outbreak	Mean	Median
1971-1980	8000	277	50
1981-1990	13 000	272	37
1991-2000*	403 000	3372	36
2001-2008	1663	83	12

**Excluding the Milwaukee outbreak, the largest outbreak resulted in 9847 illnesses (mean = 223).*

Non-Legionella *bacteria*

Shigella spp., including *S. sonnei* and *S. flexneri*, were identified as the sole pathogens in 38 (45%) of these outbreaks causing 9366 cases. *S. typhi* was responsible for two (2%) outbreaks and 270 cases. Other *Salmonella* spp., including *S. enterica* serovar Typhimurium, were identified in 16 (19%) outbreaks causing 4516 cases. *Campylobacter* spp., primarily *C. jejuni*, caused 19 (22%) outbreaks and 5608 cases. Pathogenic *Escherichia coli* caused seven (9%) outbreaks. One outbreak of *E. coli* O6:H16 resulted in 1000 cases, and five outbreaks of *E. coli* O157:H7 caused 1236 cases; a serotype was not identified in one outbreak of 1400 cases. One outbreak was caused by *Pleisiomonas shigelloides* (60 cases) and another by *Providencia* (55 cases). In the remaining four outbreaks, more than one bacterial pathogen was identified: *C. jejuni & Yersinia enterocolitica; C. jejuni & P. shigelloides*; *E. coli* O157:H7 & *C. jejuni*; *E. coli* O157:H7, *E. coli* O145 & *C. jejuni*.

Viruses

Norovirus was identified as the sole pathogen in 38 (66%) of the viral outbreaks and 14 398 cases. Hepatitis A virus caused 19 (33%) outbreaks and 600 cases. Rotavirus caused one (1%) outbreak and 1761 cases.

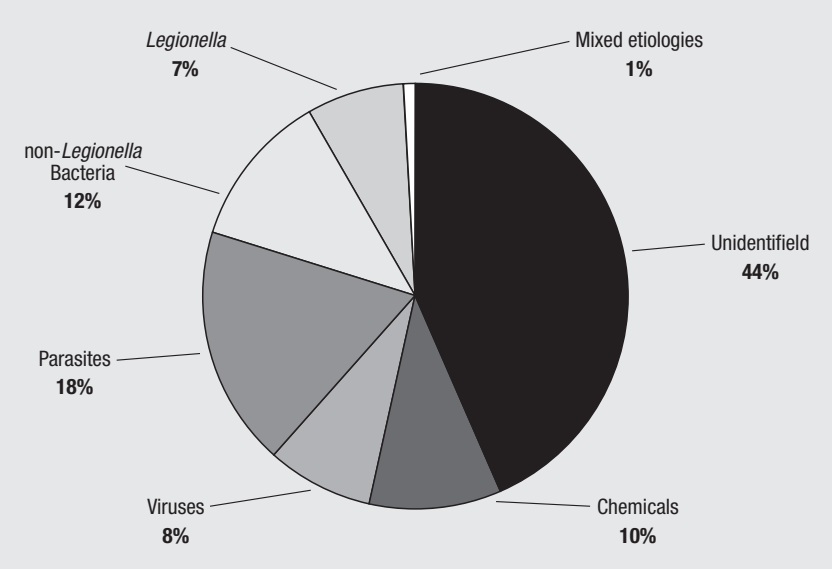

Fig. 2 | *Etiology of waterborne disease outbreaks, public water systems, 1971-2008 (no. = 733).*

Legionella

The 54 Legionellosis outbreaks resulted in 472 cases. Illness was predominately due to *L. pneumophilia* serogroup 1, but serogroups 3, 4, 5, and 6 were also identified as were *L. anisa*, *L. micdadei*, and *L. dumoffi*.

Mixed etiology

Seven (1%) outbreaks involved mixed agents. Non-*Legionella* bacteria and parasites were identified in four mixed-agent outbreaks: *C. jejuni.*, *C. lari*, *Cryptosporidium* spp. & *Helicobacter canadensis*; *Salmonella* spp. & *G. intestinalis*; *S. sonnei*, *Cryptosporidium* spp., *G. intestinalis* & *Clostridium difficile*; *C. jejuni*, *Entamoeba* spp. & *G. intestinalis*. Two outbreaks involved bacteria and norovirus. Norovirus genogroups I and II and *C. jejuni* were identified in one outbreak. Norovirus genogroup I, *Campylobacter* spp., and *Salmonella* spp. were identified in the other. The remaining mixed-agent outbreak involved *C. jejuni*, norovirus, and *G. intestinalis*. Mixed-agent outbreaks were most reported frequently during 2001-2008.

Trends

This analysis considered the 364 pathogens identified in 341 outbreaks, 73 chemical outbreaks, and 319 outbreaks of undetermined etiology for a total of 756 etiologies. The relative importance of each etiology was determined by considering the proportion of that etiology (*e.g.*, *G. intestinalis*, chemical etiology, undetermined etiology) among all of the etiologies reported during each of four specified time periods *(Figure 3)*. Three 10-year periods and one 8-year period were considered. Because outbreak statistics for 2009-2010 are likely to be available soon, eight years was chosen for the most recent period to allow for a convenient update of trends.

Most undetermined etiologies (57%) occurred during the earliest time period of 1971-1980. The percentage of undetermined etiologies then declined. During 1981-1990 and 1991-2000, undetermined etiologies represented 44% and 39% of the etiologies, respectively, and during 2001-2008, only 9% of the etiologies were of undetermined etiology. The importance of chemical etiologies varied from a low of 6% during 1981-1990 to highs of 11% and 14% during 1971-1980 and 1991-2000, respectively. During 2001-2008, 9% of all etiologies were of a chemical etiology.

Reports of parasitic agents decreased whereas reports of viruses and *Legionella* increased *(Figure 3)*. During 1971-2000, *Legionella* represented less than 5% of the etiologies, but during 2001-2008, *Legionella* represented 33% of all outbreak etiologies. Viruses represented 15% of etiologies during 2001-2008 but only 6% to 8% of the etiologies during the preceding three decades. Norovirus represented 4% to 5% of all etiologies during 1971-2000, but during 2001-2008, norovirus was identified in 15% of the etiologies *(Figure 3)*. Hepatitis A virus represented 4% or less of the etiologies during 1971-1990 but was identified in only one outbreak during 1991-2000 and none during 2001-2008. Rotavirus was identified in only one outbreak reported in 1981.

The importance of non-*Legionella* bacteria, as a group, has increased slightly. During 1971-1990, non-*Legionella* bacteria represented 11-12% of the etiologies. It has represented 16-18% of the etiologies since 1991. Before 2000, *Campylobacter* spp. represented less than 4% of the etiologies, but these bacteria were identified in 12% of the etiologies during 2001-2008. Pathogenic *E. coli*, primarily O157:H7, represented less than 1% of the etiologies before 1990 and 5% of etiologies during 1991-2000; however, only 2% of etiologies were due to *E. coli* during 2001-2008. *Shigella* spp., and *Salmonella* spp. have declined as important etiolo-

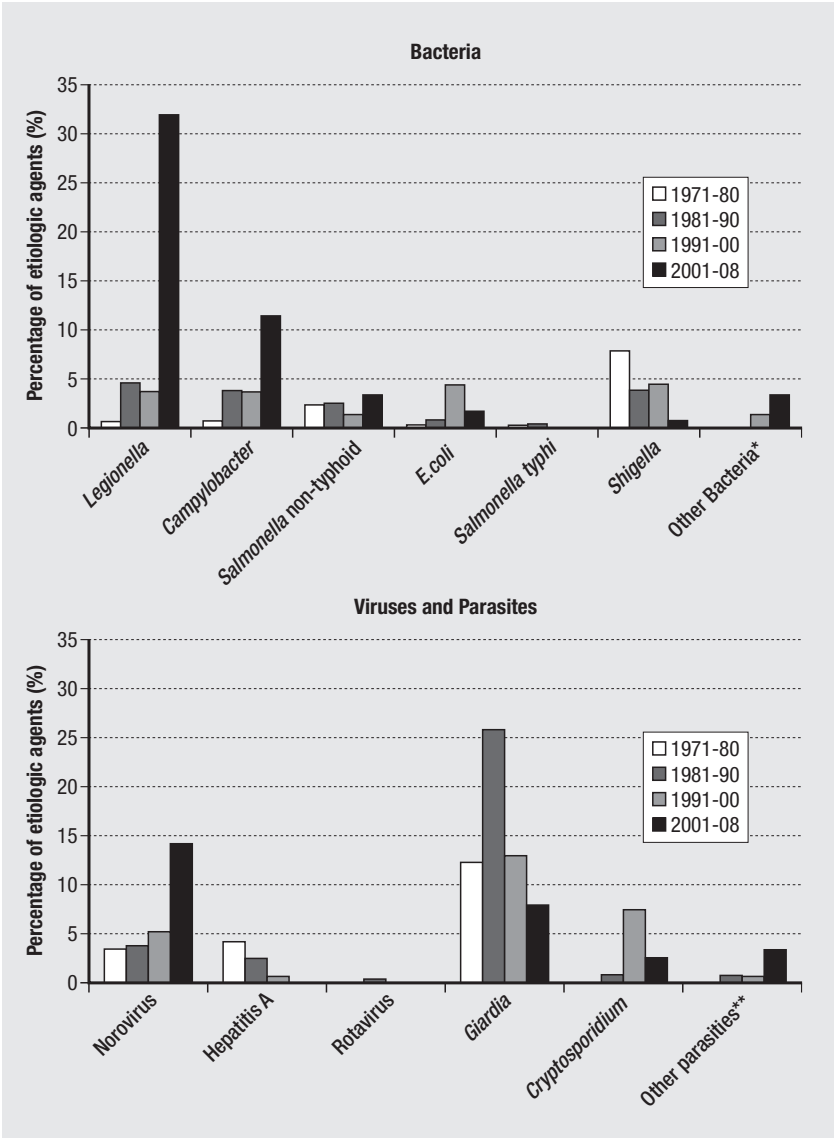

Fig. 3 | *Pathogens identified in waterborne disease outbreaks, public water systems by time period, 1971-2008.*
**Clostridium difficile, Helicobacter, Plesiomonas shigelloides, Providencia*
***Cyclospora, Entamoeba histolytica, Naegleria fowleri*

gies. During 1971-1980, *Shigella* spp. represented 8% of the etiologies, and Salmonella spp. represented 3% of the etiologies. Each represented only 1% of etiologies during 2001-2008. *S. typhi* was identified in only two outbreaks; one occurred in 1985 and the other in 1973.

Parasites have been an important etiologic agent throughout the 38-year period but their importance has diminished *(Figure 3)*.

Parasites represented 12% of the etiologies during 1971-1980 increasing to 28% and 21% of the etiologies during 1981-1990 and 1991-2000, respectively. During 2001-2008, parasites represented 15% of the etiologies. *G. intestinalis* was identified in 12%, 26%, and 13% of etiologies during 1971-1980, 1981-1990, and 1991-2000, respectively but only 8% of the etiologies during 2001-2008. *Cryptosporidium* spp. was identified in 8% of etiologies during 1991-2000 but only 3% of the etiologies during 2001-2008.

Minor etiologic agents include *P. shigelloides* as the sole pathogen in one outbreak and one of two pathogens in another outbreak, both reported during 1991-2000. *Y. enterocolitica* and *C. difficile* were identified along with other agents in two separate outbreaks during 2001-8. *Providencia*, *N. fowleri*, and *C. cayetanensis* were also identified during 2001-2008, each causing one outbreak.

Water system deficiencies

Investigators identified 720 deficiencies during 1971-2008. A single water system deficiency or source of contamination was found in 685 outbreaks. Two deficiencies were identified in 16 outbreaks and three deficiencies were identified in one outbreak. In almost all (94%) of the multiple-deficiency outbreaks, investigators found contamination from distribution system mains, storage facilities or premise plumbing (*e.g.*, cross-connections or backflow) in addition to contamination from untreated or inadequately

treated water. In 30 outbreaks, engineering and environmental information was inadequate to identify a deficiency.

The importance of each major deficiency category was considered for the entire 38-year period *(Figure 4)*.

Inadequate or no treatment of ground water (49%) and surface water (20%) were the two most frequently reported deficiencies during the 38-year period *(Figure 4)*. Outbreaks were also caused by contamination of premise plumbing (16%) and contamination of the distribution system (10%). Premise plumbing refers to pipes and storage facilities within a building or house. The distribution system refers to pipes and storage facilities located outside a building or before entry to the property line. For community systems, the distribution system is under the control of the water utility. Contamination of water at its point of use (*e.g.*, contaminated hose bibs or water storage containers) was a minor cause of outbreaks (1%).

In the five of the outbreaks attributed to inadequate water treatment, investigators did not identify a water source in one outbreak and in four outbreaks both ground and surface water sources were used. When both the water source and deficiency are considered in an analysis, the four outbreaks in mixed-source systems were considered as surface water outbreaks. The one outbreak with an unidentified water source was included among those with insufficient information.

Inadequate water treatment

During 1971-2008, no treatment of ground water (25%; no.= 189) was reported more frequently than no treatment of surface water (2%; no. = 16). Information about the source of contamination or contributing factors was provided for 110 of the untreated ground water outbreaks. Investigators identified sewage overflow or seepage into the ground water in 44% of these 110 outbreaks. In an additional 18% of outbreaks, contaminants from unspecified sources entered wells developed in limestone or fissured rock aquifers. Ground water was contaminated during heavy rains and flooding in 22% of the outbreaks associated with untreated ground water. Improper construction and location too near a stream allowed contamination by surface water in 8% and 4% of these outbreaks, respectively. Chemical or pesticide contamination was identified in the remaining 4% of these outbreaks.

Inadequate treatment of ground water (24%; no. = 177) was also reported more frequently than inadequate treatment of surface water (17%; no. = 131). Details of the treatment deficiencies were available for 174 of these ground water outbreaks. Almost all of these outbreaks occurred in systems where disinfection was the only treatment for pathogens and were due to either inadequate (40%) or interrupted (49%) disinfection. Other treatment deficiencies for these 174 ground water outbreaks included inadequate control of chemical feed other than disinfection (7%), inadequate control or operation of filtration facilities (2%), and improper filter design (1%).

Details of the treatment deficiencies were available for 130 of the 131 outbreaks caused by the inadequate treatment of surface water. Most deficiencies were associated with disinfection as the only treatment, either inadequate (51%) or interrupted (15%) disinfection. Other surface water treatment deficiencies included inadequate control of filtration (26%), inadequate control of chemical feed other than disinfection (4%), bypass of filtration (2%), and inadequate or interrupted disinfection with filtration (1%).

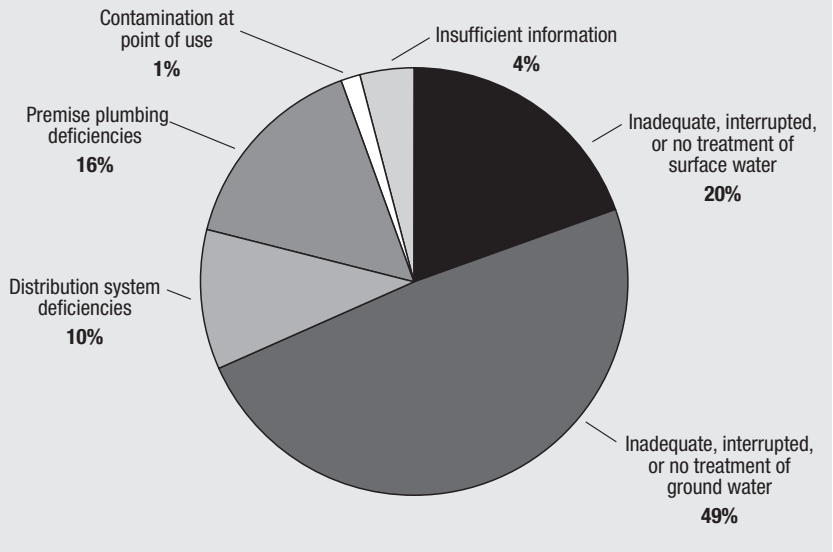

Fig. 4 | *Deficiencies identified in waterborne disease outbreaks, public water systems, 1971-2008 (no. = 750).*

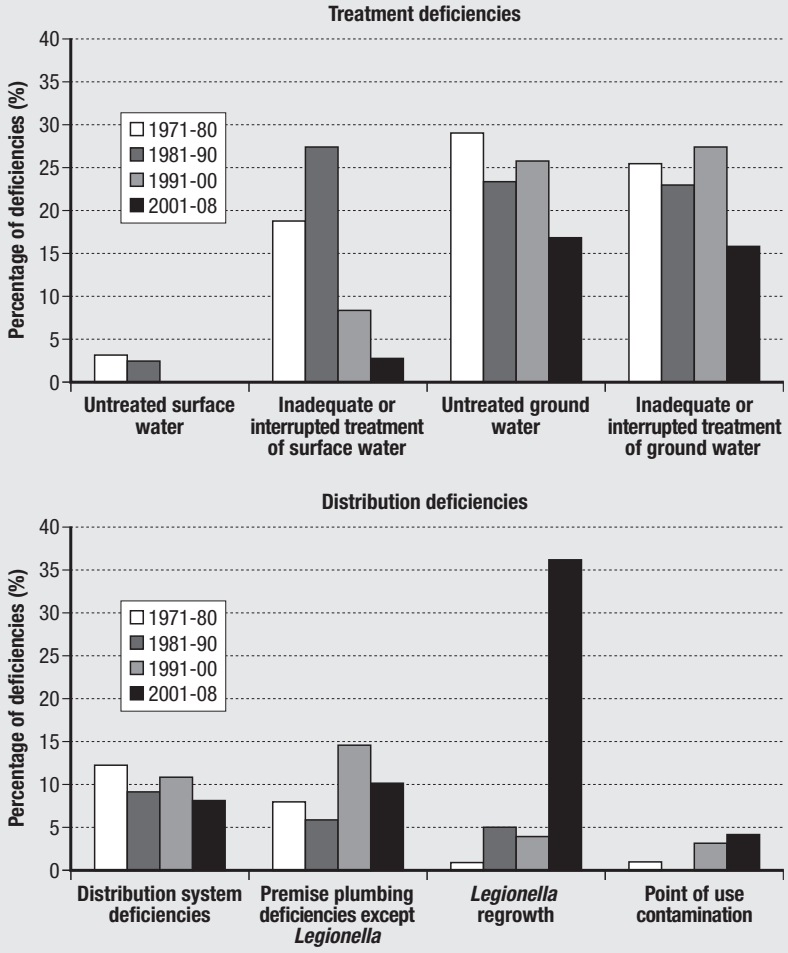

Fig. 5 | *Deficiencies identified in waterborne disease outbreaks, public water systems by time period, 1971-2008.*

Distribution system and premise plumbing deficiencies

Most (55%) distribution system deficiencies during 1971-2008 were due to cross-connections or backflow events. Other distribution system deficiencies included contamination of storage facilities (19%), broken or leaking water mains (15%), and contamination of mains during construction, repair, or flushing (9%). *Legionella* regrowth was identified in 47% of the outbreaks where premise plumbing deficiencies were reported. Also identified were: cross-connections or backflow events (30%), corrosion byproducts of plumbing systems or drink-mix machines (20%), and contamination of service lines or in-building storage facilities (3%).

Trends

The relative importance of each deficiency was determined in the same manner and for each of the same four time periods considered for etiologies. The analysis considered 750 deficiencies, which includes the 30 outbreaks with unidentified deficiencies *(Figure 5)*. Unidentified deficiencies represented 2% to 4% of the deficiencies reported during 1971-1990 and 6% of the deficiencies reported during 1991-2008.

The majority (55%) of deficiencies during 1971-1980 involved the use of contaminated ground water that received either no treatment (29%) or inadequate treatment (26%). The use of untreated ground water continued to be an important deficiency being responsible for 24% of deficiencies during 1981-89, 26% during 1991-2000, and 17% during 2001-2008. The inadequate treatment of ground water also continued to be important; it was responsible for 23% of deficiencies during 1981-90, 28% during 1991-2000, and 16% during 2001-2008.

The use of untreated contaminated surface water was responsible for less than 3% of the deficiencies during 1971-1990. Afterwards, the EPA regulations required treatment for surface water sources. The inadequate treatment of surface water was responsible for 19% and 27% of all deficiencies during 1971-1980 and 1981-1990, respectively. As more attention was given to surface water treatment, the importance of this deficiency was diminished; only 8% and 3% of the deficiencies were due to the inadequate treatment of surface water during 1991-2000 and 2001-2008, respectively.

Premise plumbing deficiencies have increased in importance, and distribution system contamination con-

Table 4 | *Percentage of etiologic agents responsible for waterborne disease outbreaks, public systems by time period, 1971-2008*

Etiology	1971-1980 (no. = 278)	1981-1990 (no. = 232)	1991-2000 (no. = 128)	2001-2008 (no. = 95)
Legionella	1%	5%	4%	38%
non-*Legionella* bacteria	12%	11%	15%	11%
Viruses	8%	7%	6%	14%
Parasites	12%	27%	20%	11%
Chemicals	11%	6%	14%	10%
Unidentified etiology	56%	44%	40%	10%
Mixed etiology	0	< 1%	1%	6%
total	100%	100%	100%	100%

tinues to be important. The contamination of premise plumbing with *Legionella* was responsible for 36% of the deficiencies during 2001-2008. During the same time period, other pathogens or chemicals were responsible 10% of the deficiencies. Contamination of distribution system pipes and storage facilities was identified in 8% of the deficiencies during 2001-2008 only slightly less than the 9% to 12% in previous time periods.

Outbreak risks

Although the relative importance of an outbreak deficiency during each decade is informative, this measure does not take into account changes that may have occurred in water sources and treatment. This is important when assessing risks associated with the treatment for surface water sources. In 1986, information became available about the number of public water systems that used surface water and the type of treatment that was employed [22]. At that time there were 9527 public systems that used surface water. The number increased to 13 608 systems in 1998 [36] and 14 371 systems in 2010 [12]. Outbreak rates computed for the period 1971-1985 showed that community water systems with disinfection as the only treatment for surface water had an almost 8-fold higher outbreak risk than systems with both disinfection and filtration [37]. Outbreak risks for systems using untreated surface water were similarly high.

For this article, I computed outbreak risks for public systems using surface water during 1981-2008 using water system inventory information available from the EPA [22, 36, 38]. These risks consider only infectious disease outbreaks; chemical outbreaks were excluded from the analysis. Both the number and risk of outbreaks declined for all three types of surface water systems *(Table 5)*. Outbreak risks for public systems using untreated or disinfected-only surface water during 1981-1990 were 5 to 6-fold higher than risks for systems using filtered and disinfected surface water. During 1991-2000, the outbreak risk for disinfected-only surface water systems declined considerably but was still almost 2-fold higher than for filtered systems. The outbreak risk for filtered surface water systems decreased to about half of the risk seen during 1991-2000.

During 1981-1990, six outbreaks were reported in public systems using untreated surface water systems and 39 outbreaks in systems providing only disinfection. The most recent outbreak associated with untreated surface water was reported in 1990. There were five outbreaks associated with unfiltered, disinfected surface water systems during 1991-2000 and no outbreaks have been reported in disinfected only surface water systems since 1997. Outbreaks in filtered, disinfected surface water systems decreased from 22 outbreaks during 1981-1990 to five outbreaks during 1991-2000 and three outbreaks during 2001-2008. Two of the three outbreaks associated with filtered systems since 2001 occurred in small non-community systems that provided water to campgrounds and resulted in 60 illnesses. The third outbreak of 1663 cases of gastrointestinal illness occurred in a community water system supplied by surface water conventionally treated with coagulation, settling, filtration, and disinfection [10]. Investigators identified numerous deficiencies in the operation and maintenance of disinfection and filtration facilities including the recycling of untreated filter backwash water. Filter

Table 5 | *Outbreak risks associated with inadequate treatment, public water systems using surface water, 1991-2008*

	Infectious disease outbreaks per year per 10000 systems		
Water source and treatment	1981-1990	1991-2000	2001-2008
Surface water, untreated	18.99	0	0
Surface water, disinfection only	16.05	1.08	0
Surface water, filtration and disinfection	3.18	0.56	0.31

performance was adversely affected when backwash water was routed directly to the treatment flow bypassing the recovery basin and disinfection.

Less attention has been paid to outbreak risks in ground water systems, but it is also important to consider these because public systems using ground water have decreased from 156 768 in 1998 [36] to 138 527 in 2010 [12]. The number of infectious disease outbreaks associated with untreated and inadequately treated ground water also decreased from 61 during 1991-2000 to 30 during 2001-2008 with approximately half of the outbreaks in untreated ground water systems. However, the outbreak risks for untreated ground water systems remained relatively stable with rates of 0.29 and 0.21 outbreaks per year per 10 000 systems in 1991-2000 and 2001-2008, respectively. Outbreak risks associated with treated ground water systems declined slightly during the same time periods from 0.68 to 0.40 outbreaks per year per 10 000 systems. All except three outbreaks during the past 18 years were due to inadequate or interrupted disinfection as the only treatment for ground water. Three outbreaks occurred in filtered and disinfected ground water systems; one in 1991-2000 and two in 2001-2008.

DISCUSSION

The US waterborne disease outbreak surveillance system has largely been successful because of the continued cooperation of public health professionals at the EPA, CDC and various state agencies. This cooperation is necessary because the responsibility for establishing national drinking water regulations and conducting the accompanying health effects and water treatment research resides within the EPA, whereas the responsibility for disease surveillance resides within CDC. In addition, most states have a similar arrangement where environmental protection agencies are responsible for ensuring the safety of drinking water while state public health agencies are responsible for disease surveillance and outbreak investigation. Even when both of these responsibilities reside within the same state agency, several different bureaus may be involved. In addition, outbreak investigation requires professionals with differing technical expertise, some of which may be provided from sources outside the government. Continued leadership at the federal and state levels is necessary to ensure that outbreaks are detected, investigated, and reported.

This review of the surveillance statistics describes the causes of outbreaks associated with public water systems and the changes that have occurred in the etiologic agents, risk factors, and major deficiencies during the past 38 years. Increased reports of outbreaks in the early 1980s emphasized that microbial contaminants remained a health-risk challenge for suppliers of drinking water. Analyses of the causes of these outbreaks helped focus attention on water system deficiencies, especially the high risk of outbreaks associated with unfiltered surface water systems, and this provided the stimulus for the research that formed the basis for water system improvements. Research included studies that demonstrated the removal of *Giardia* cysts and *Cryptosporidium* oocysts by water filtration and inactivation of parasites, viruses, and bacteria by disinfectants [39, 40]. All of the EPA rules to reduce microbial risks contained a discussion of the waterborne outbreak statistics that showed the need for intervention as well as the remedies suggested by the research studies. Although surveillance statistics have provided important information which is summarized below, readers should be aware that they represent only a portion of the incidence of outbreaks. Not all outbreaks are recognized or reported [41] and outbreak statistics do not reflect the burden of endemic waterborne illness [42, 43].

Increased identification of etiologic agents

The decreased proportion of outbreaks associated with undetermined etiologies is likely due to newly developed and improved laboratory diagnostic tools to identify etiologic agents in clinical specimens and water samples (*e.g.*, detection of norovirus). A contributing factor may be early outbreak detection which allows for a more timely collection of clinical specimens and water samples. Both timely collection and improved laboratory analyses are important to identify waterborne pathogens of emerging importance. The continued collaboration of state public health and drinking water regulatory agencies is required to ensure the early recognition of an outbreak and its investigation. It is especially important that prior arrangements are made to ensure adequate laboratory support during outbreak investigations.

Decreased reports of outbreaks in public systems

The number of outbreaks associated with public water systems has decreased, especially since the peak reporting in the early 1980s. This decrease is likely the result of national drinking water regulations and improved water system management, operation, and treatment practices. Improvements were largely due to the cooperative efforts of the EPA, public water systems, the American Water Works Association, and the Association of State Drinking Water Administrators. An example of cooperative efforts is the Partnership for Safe Water, whose objective is to improve water plant performance, emphasizing the importance of design, operation, and monitoring of filtration and disinfection [44].

Decreased importance of inadequately treated surface water

There has been a decrease in both the proportion of deficiencies and outbreak risks associated with inadequately treated surface water in public systems. The decrease is associated with the promulgation

of EPA regulations and water treatment improvements to reduce risks in surface water sources from *Giardia* and *Cryptosporidium*. During 2001-2008, no outbreaks were reported in unfiltered surface water. The three outbreaks associated with filtered water systems during the same period, emphasize the importance of proper operation and maintenance of disinfection and filtration processes, especially for small public systems.

Continued importance of inadequately treated ground water

There has been no significant reduction in outbreak risks or the proportion of outbreaks associated with untreated or treated ground water. The inadequate or no treatment of ground water accounted for one-third of all deficiencies reported during 2001-2008. Sixteen outbreaks were associated with public systems that provided treatment of ground water during the past eight years. The over feeding of sodium hydroxide was responsible for three outbreaks. Eleven outbreaks occurred in disinfected ground water systems; in six outbreaks, disinfection was interrupted and in five disinfection levels were inadequate.

In one of two outbreaks where filtration was provided, the filter was not designed to remove cysts and oocysts. The second outbreak involved a spring under the direct influence of surface water. After installing a slow sand filter, inadequate time was allowed for formation of a *schmutzdecke* biological layer on the surface of the filter, and this resulted in poor removal of pathogens. These ground water outbreaks are a reminder not only of the potential for contamination of source water but also the need for increased attention to design of treatment facilities and their proper operation. When provided, treatment should be adequate to cope with anticipated source water contamination and operated without interruption. Implementation of EPA's Ground Water Rule [26, 27] should help decrease risks for ground water systems.

Increased importance of premise plumbing contamination

The increased proportion of outbreaks associated with premise plumbing deficiencies is largely due to *Legionella* regrowth. *Legionella* can grow within premise plumbing water pipes, and prevention of outbreaks requires careful attention to maintaining water systems according to published guidelines [45]. During 2001-2008, acute respiratory illness was reported in 38% of the outbreaks, all associated with the amplification and dissemination of *Legionella* through premise plumbing, pipes, and storage infrastructure. Two-thirds of the outbreaks occurred in hospitals, nursing homes, and other health care settings, demonstrating the ability of *Legionella* to colonize the biofilms frequently found inside the large, complex plumbing systems of these facilities.

Continued importance of distribution system contamination

The proportion of outbreaks associated with distribution system deficiencies is similar to premise plumbing deficiencies when legionellosis outbreaks are excluded from the analysis. Increased attention to this problem is warranted because of the continuing occurrence of outbreaks as well as studies that show the potential for contamination through low pressure events [45] and need for infrastructure improvements [46, 47].

CONCLUSIONS

This review illustrates the importance of a waterborne disease outbreak surveillance system.

Surveillance statistics collected during the past 38 years in the United States have helped identify important water system deficiencies, guiding prevention efforts at the national, state, and local levels.

Surveillance statistics have also demonstrated the effectiveness of prevention efforts. Waterborne outbreak risks associated with the treatment of surface water have declined significantly.

However, outbreaks due to other water system deficiencies still occur, and continued surveillance is warranted. Collaboration must be maintained among current government and nongovernment participants for an effective US surveillance system.

Although the CDC has the primary responsibility for waterborne outbreak surveillance, the EPA should continue to be an active partner because of its regulatory and research responsibilities.

Timely knowledge about the etiologies and causes of waterborne outbreaks has been, and will continue to be, important in establishing research priorities and recommending preventive measures.

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Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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Vaccine preventable viral diseases and risks associated with waterborne transmission

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Abstract. Rotavirus and poliovirus are paradigmatic viruses for causing major diseases affecting the human population. The impact of poliovirus is remarkably diminished because of vaccination during the last half century. Poliomyelitis due to wild polio currently affects a limited number of countries, and since 2000 sporadic outbreaks have been associated to neurovirulent vaccine-derived polioviruses. Conversely, rotavirus is presently very diffuse, accounting for the largest fraction of severe gastroenteritis among children <5 years-old. Vaccination towards rotavirus is still in its dawn, and zoonotic strains contribute to the emergence and evolution of novel strains pathogenic to man. The environment, particularly surface water, is a possible vehicle for large transmission of both viruses, but environmental surveillance of circulating strains can help promptly monitor entry of new virulent strains into a country, their shedding and spread.

Key words: rotavirus, poliovirus, vaccine, waterborne transmission, surveillance.

Riassunto *(Malattie virali prevenibili con la vaccinazione e rischi associati alla trasmissione idrica)*. Rotavirus e poliovirus sono virus paradigmatici nel causare malattie importanti della popolazione umana. L'impatto dei poliovirus è diminuito marcatamente durante mezzo secolo di vaccinazione. La poliomielite dovuta a polio selvaggio affligge solo un limitato numero di paesi, e dal 2000 epidemie sporadiche sono state associate a poliovirus nerurovirulenti vaccino-derivati. Al contrario, rotavirus è oggi molto diffuso, essendo implicato nella maggiore parte dei casi di gastroenterite grave nei bambini di meno di 5 anni. La vaccinazione contro i rotavirus è ancora agli albori, e ceppi zoonotici contribuiscono all'emergenza ed evoluzione di nuovi ceppi patogeni per l'uomo. L'ambiente, in particolare le acque superficiali, è un possibile veicolo per la trasmissione di entrambi i virus, ma la sorveglianza ambientale dei ceppi circolanti può aiutare a monitorare prontamente l'ingresso di nuovi ceppi virulenti in un paese, il loro rilascio e diffusione.

Parole chiave: rotavirus, poliovirus, vaccino, trasmissione idrica, sorveglianza.

INTRODUCTION

Water represents an important vehicle for transmission of viruses responsible for a wide range of diseases in humans and animals, particularly viruses colonizing the gastrointestinal tract. Intestinal agents such as poliovirus and other enteroviruses, norovirus or rotavirus can be shed with stools at high concentrations for days or weeks. These may heavily contaminate the environment, reaching watercourses and other surface water basins, and eventually enter the potable water distribution pipelines, or crops, soft fruit and vegetables, or water-filtering seafood, posing a risk for possible epidemic outbursts of disease.

Viruses infecting the intestine of man or animals are normally quite resistant in the harsh condition of the environment, also due to an envelope-free capsid made of only proteins, which provides efficient protection for the viral genome.

In the absence of effective therapeutic treatments for most viral diseases, and in consideration of the large epidemic potential of many of the viral agents shed with stools, vaccination is the most reliable approach that can be adopted to halt or limit viral diffusion through the susceptible populations. However, the great genetic variability of many viruses, often resulting in the emergence of distinct serotypes, makes the development of largely effective cross-reactive vaccines problematic. Finally, the globalization with the consequent massive travelling of people, animals and goods between different areas of the world, has further enhanced the emergence and spread of novel viruses in naïve populations, sometimes showing full or mixed characters of agents from the animal world.

In this review, we report studies on the impact of vaccination in the distribution of poliovirus and ro-

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tavirus, two typical enteric viruses able to severely affect humans, and the role of environmental waters in strain circulation among susceptible individuals.

Rotavirus

Rotavirus was discovered in the early '70s, and soon identified as the major etiological agent of infectious acute gastroenteritis in childhood [1]. Today, rotavirus remains the single most important cause of morbidity among pediatric patients with diarrhea worldwide, more than any other viral, bacterial or parasitic organism, and is estimated to cause approximately 450 000 deaths per year, predominantly in developing countries [2]. It is believed that every single child in developed countries contracts rotavirus at least once during its first 2½ years of life, although only a lesser number of subjects develop diarrhea severe enough to require hospital admission and medical intervention, including rehydrating therapies.

Structure, proteins and genome

Rotavirus represents a genus of the Reoviridae Family [3], and derives its name from its wheel-like (rota, in Latin) shaped virion, approximately 75 nm in diameter. The virion is rather complex, and the variability in the functions and antigenicity of its proteins largely influences the host-pathogen relationships, including host range restriction.

The capsid is made of three concentric protein layers, engulfing 11 segments of double-stranded RNA (dsRNA) with molecular size ranging between approximately 670 through 3300 base pairs (www.iah.bbsrc.ac.uk/dsRNA virus proteins/Rotavirus.htm) [3]. This complete form of infectious rotavirus is also known as TLP (triple-layered particle).

The outermost rotavirus shell comprises two proteins, VP7 and VP4, containing epitopes which elicit neutralizing protective antibodies. The glycosylated protein VP7 forms a continuous layer of trimers, and represents the major neutralization antigen, specifying the virus G-serotype [3]. The VP7 layer is crossed by 60 spike-like projections of the protein VP4, which determines the P-serotype, and is cleaved by pancreatic trypsin yielding two fragments, VP8* and VP5*, that results in profound conformational modifications and enhanced infectivity. VP4 is the viral protein responsible for attachment to the susceptible cells, and has been recently shown to bind histo-blood group antigens (HBGA) in a serotype-specific manner, that might explain differential host susceptibility to infection [4].

The inner rotavirus shell is only made of the VP6 protein, recognized as the "group antigen" of rotavirus which, and due to its ample antigenic conservation between distinct G- and P-serotypes it is conveniently used for antigen-based diagnostics [5]. Based on VP6, 7 groups of rotaviruses are distinguished designated A through G, among which group A contains by far the most important viral strains pathogenic for humans, and many other animal species. Man is also infected with group B and C rotaviruses, known for being implied in large water-borne outbreaks in Asia and for causing sporadic or epidemic gastroenteritis cases in children and adults, respectively [3].

In addition to 3 further structural proteins (VP1-3), the 11 segments rotavirus genome also codes for 6 non-structural proteins (NSP1-6), involved in either RNA transcription or progeny virus maturation [3]. Importantly, NSP1 and NSP3 appear to have specific roles in rotavirus virulence and possibly host range restriction. NSP4 is functional to virus final morphogenesis [3], and is also acting as an enterotoxin, being able to induce diarrhea in the infant mouse pathogenesis model [6], and likely exerting its effects *in vivo* by interacting with the luminal enterochromaffin (EC) cells and the enteric nervous system (ENS) [7].

Serotypes, genotypes and immunity

Group A rotaviruses can be differentiated into serotypes based on reactivity with hyperimmune sera or neutralizing monoclonal antibodies directed at either VP7 or VP4 [8]. Since the two genes encoding VP4 and VP7 segregate independently, a binomial serotype system was adopted to identify strains, defined as GxPy serotypes. However, viral characterization is normally conducted by more friendly molecular approaches using semi-nested RT-PCR and panels of genotype-specific oligonucleotide primers [9]. Genotyping is considered to be a valid proxy for serotyping, and is adopted universally. As a result, a large amount of data is being accumulated concerning rotavirus genotypes and serotypes circulating worldwide [10, 11], which is valuable to confirm the adequacy of current vaccines in relation to emergence and evolution of viral strains [12, 13]. Currently, at least 27 different G-types and 35 P-types are recognized among human or animal worldwide [14], and increasing evidence of zoonotic transmission of animal rotaviruses to humans, involving reassortment mechanisms during dual infection, has strengthened the threat of novel rotavirus strains emergence from domestic and wild animals with respect to vaccine efficacy [15]. It underlines the need for surveillance of circulating rotavirus strains in order to identify emerging or reassortants strains with unusual serotype.

Due to the high death toll and economic burden of rotavirus disease worldwide, vaccine strategies for disease control were implemented through joint efforts of scientists, industry and public health authorities as early as in the mid-90's [12, 13].

Two live-attenuated vaccines (RotaTeq®, by Merck; Rotarix®, by GlaxoSmithKline) have been successfully introduced in a growing number of countries since 2006 [16], and although they are respectively pentavalent and monovalent they appear to be largely protective against co-circulating rotavirus genotypes in human populations [17]. However, some lower efficacy of vaccination is reported in less developed countries [17], possibly related to unsatisfactory herd immunity established against uncommon rotavirus genotypes

circulating in these areas [11, 18]. Vaccination is also considered an important line of defense for several animal species with an economic value.

Protection to rotavirus disease can be afforded by both symptomatic and asymptomatic natural infection, as well as by vaccination [13, 19].

Also, animal strains are normally less pathogenic for humans than they are in their species of origin, a knowledge that drove towards a Jennerian approach to rotavirus vaccines in childhood [20].

However, early volunteer studies and field investigations demonstrate that at least some strains deriving from sick children can re-infect adults despite the presence of pre-existing immunity [21], although often in the absence of symptoms. This is confirmed by community studies showing that rotavirus infection was shed by mild sporadic cases of diarrhea not necessitating hospitalization or asymptomatic subjects, spanning all ages [22]. In the UK, rotavirus-specific IgM was detected in the normal adult population with no seasonal trend [23], suggesting constant circulation of the virus among adults independent on the winter peak of disease typical for children. Although immunity to rotavirus gastroenteritis has been considered to be life-long, adults might play a role of healthy carriers and reservoir for rotavirus [24].

Epidemiology of rotavirus infection and disease

Rotavirus transmission follows the fecal-oral route, and is mainly associated to direct inter-human passage. However, infectious viruses are shed in large amounts into the environment by both humans and animals, contaminating water, food and feed [3, 25]. Rotavirus pediatric cases show a seasonal trend, with an epidemic peak in cooler, drier months, particularly in countries with a temperate climate [26], that might involve a longer persistence of live virions in the environment in particular conditions. However, in addition to environmental conditions favoring virus survival, differences in rotavirus seasonality between areas may be also ascribed to individual country birth rate and virus transmission dynamics [27].

The age range associated to higher risk of severe rotavirus gastroenteritis is 6 to 24 months [28], but older children can also need medical care or hospitalization [29]. The estimated number of global rotavirus diarrhea cases approaches 110 million yearly, 2 million of which necessitate admission to hospitals [2, 30]. Although most of fatal cases are restricted to developing areas *(Table 1)*, a high rotavirus burden is recorded in industrialized countries; as an example of the magnitude, before mass vaccination was introduced in 2006 in the US, rotavirus caused in this country 410 000 physician visits, 70 000 hospitalizations, and 272 000 emergency department visits per year, costing the society more than $1 billion [30].

The main symptoms during rotavirus infection are diarrhea and vomiting, and disease can vary from mild disease up to severe dehydration, osmotic shock and death. On a clinical basis, the severity of cases is conventionally determined by the Vesikari scoring system, that assigns between 0-20 score considering several factors, such as duration of diarrhea, number of stools in 24 hours, vomiting duration, number of vomiting episodes in 24 hours, maximum fever, medical visits, and treatment (none, outpatient and hospitalization) [28]. Asymptomatic rotavirus infection also occurs frequently in both children and adults, possibly related also to partial immune protection following earlier infection. These data highlight the risk that rotavirus shed by healthy people may be transmitted to susceptible non-immune subjects, as the newborn, directly or through environmental and/or foodstuff contamination circuits [31, 32].

The mechanisms and duration of protection in rotavirus infection are not completely understood, particularly regarding the extent to which different serotypes or "genotypes" of rotavirus underpin antigenic diversity that may affect the immune system reaction against infection with different strains [33, 34]. It also raises problems in evaluating the risks of zoonotic transmission of animal strains, and as a consequence the risks for human health associated with environmental contamination with animal feces and manure, animal farming effluents, in addition to human sewage. Since group A rotaviruses infect many animal species, including domestic animals and pets, susceptible subjects are also exposed to a large variety of strains of animal origin [15, 35]. Reassortment of genome segments during co-infection with several RV strains is crucial in favoring adaptation of zoonotic animal rotavirus strains in man, via environmental or food-borne transmission [33, 36].

Rotavirus zoonotic transmission

Despite some constraints in rotavirus replication and spread between different animal species or hu-

Table 1 | *Number of rotavirus diarrhea deaths among children in countries with highest mortality rates, 2008*

Country	No. of deaths	Percent on all deaths
India	98 621	21.8*
Nigeria	41 057	30.8
Pakistan	39 144	39.5
Democratic Republic of Congo	32 653	46.7
Ethiopia	28 218	52.9
Afghanistan	25 423	58.6
Uganda	10 637	60.9
Indonesia	9 970	63.1
Bangladesh	9 857	65.3
Angola	8 788	67.2
Total	304 368	

**Percentage of rotavirus specific deaths over total deaths for all causes The total of deaths in the 10 countries reported in the Table represents 67.2% of the 453 000 children dead with rotavirus infection in that year. Source: WHO IVB (http://www.who.int/immunization_monitoring/burden/rotavirus_estimates/en/index.html).*

mans, interspecies and more specifically zoonotic transmission of rotaviruses is now recognized to occur frequently, sometimes resulting in overt disease in the heterologous species [15, 35]. As an example, G9 rotavirus is thought to have possibly originated from swine, being a rare cause of infantile gastroenteritis in US in 1983-1984, expanding significantly among symptomatic children in this and other countries after a decade, and becoming one of the 5 commonest human rotaviruses today. The P[6] gene normally found in swine was also circulating in the same period, and when G9 emerged throughout the world G9P[6] strains were frequently observed [37]. The occurrence of G9P[6] and G9P[8] in man, and other findings of typically human G types in animals are altogether suggestive of natural genetic reassortment between human- and porcine strains, eventually leading to a novel globally widespread virus type [11, 37].

Also because of improved sequencing and whole genome genotyping analysis of rotavirus to explore rotavirus evolution, it is now possible to identify emerging zoonotic strains with a possible potential for rapid global spread, also in consideration of an increasing herd immunity by extending vaccination [13]. Using these approach, G8P[8] and G8P[6] strains identified in children with diarrhea in the Democratic Republic of Congo in 2003 [36] could be studied in detail, demonstrating for 9 of their genes a close evolutionary relationship with rotavirus strains belonging to the DS1-like (G2P[4]) sub-group, and suggesting at least three, and possibly four, consecutive reassortment events involving both DS1-like and Wa-like human rotaviruses and more animal strains of bovine (G8) and swine origin.

This process of "humanization" following zoonotic transmission may further proceed generating new virus reassortants, as was shown in two distinct G8P[8] and G8P[4] rotaviruses reported in 2006 and 2009 in Europe, showing partial or little similarity with the DRC strains and close phylogenetic links with other common human rotavirus circulating in Europe belonging to G types other than G8 [38, 39]. One of these latter strains, G8P[8] with a full Wa-like genome, unexpectedly became predominant among children with severe gastroenteritis in Croatia in 2006, suggesting that its emergence was [36, 38] favored by an unusual gene repertoire [13, 17, 36].

There is increasing evidence that some animal species may play a relevant role of reservoir of rotavirus strains transmitted zoonotically. Close genetic relatedness between strains of different origin suggests that ruminants and ungulates may be the reservoir of G6 rotaviruses for humans [15]. Besides the G9 strains reported above, the swine is also regarded as a possible reservoir of G3, G5, G12 and P[6], P[16], P[19] rotaviruses.

The emerging G12 genotype increasingly reported in humans worldwide are also thought to have originated from swine establishing in man after animal-human reassortments [15, 18].

Also the rabbit has been proposed to harbor rotaviruses with similar characteristics to strains found in gastroenteritis cases in children, as well as pet animals like dogs and cats [15, 33]. All of these animal species and others may take part in generating novel zoonotic strains evolving across multiple animal reservoirs, as far as different animal species get into direct contact or share a same environment or vehicles of fecal contamination.

Rotavirus and the environment

The environment, and more specifically surface or recreational waters, can be contaminated by introduction of fecal pathogens, including rotavirus, via sewage spill-out, which may sporadically become massive as a consequence of flood, sewage-treatment plants failure, pipeline leakage, and others. These aspects are reviewed in more detail in a separate article of this same issue, and will not be presently examined in further detail.

Occurrence of rotavirus in environmental water and its association to a community waterborne gastroenteritis outbreak [40] were established even before sensitive molecular detection methods became available.

It has long been known that rotavirus passing through urban waste-water treatment plants is only subject to partial viral load reduction before proceeding into receiving environmental water [41, 42]. Also, it is noteworthy that rotavirus is stable in contaminated food, fomites and environmental matrices, and is resistant to disinfection [43]. Persistence of rotavirus in surface or drinking waters as well as in food or on surfaces is remarkable [44-46], and it is widely known that irrigation water contaminated with feces or organic fertilizers can cause pre-harvest contamination of fruits and vegetables with enteric viruses in general [44, 45]. For these reasons, quantitative risk assessment models have been proposed for water, wastewater and manure to the food safety operators with the final aim of preventing contamination of the food chains with rotavirus and other viral pathogens [47].

Besides crops, fecal shedding of rotavirus into surface waters would also have an impact on natural banks or farming sites of mollusk seafood. In Italy, as many as 18% of natural bank mussels were found to be positive for rotavirus [46], and a relevant rate of natural contamination of seafood with rotavirus was confirmed in several other field studies conducted in other countries [48].

One aspect that has not yet been analyzed in sufficient details is the asymptomatic infection of adults and older children, since they can release into the sewage rotavirus strains that are not necessarily the same shed during infantile diarrhea. In fact, stools and virus shed from patients of younger age are normally collected on diapers, which are not disposed into the sewage pipeline, and thus may not re-enter the environmental contamination route as do adult feces. Information from rotavirus genotyping studies applied to sewage samples is very limited, yet it suggests that significant differences may exist between viral types released into sewage and those

detected in symptomatic pediatric cases. Whether this may imply diminished or increased risk of infection of susceptible children is unclear, also because surface waters and water-contaminated vegetables or seafood are hardly a good vehicle for direct viral transmission to infants. However, environmental contamination would surely help rotavirus circulate through the adult population as in the case of noroviruses and hepatitis A viruses, whose clinical effects are remarkable permitting identification of sporadic cases, outbreaks, and their attribution to environmental pollution. Eventually, asymptomatic infected adults may unconsciously transmit rotaviruses to the susceptible children within households or institutions.

In some studies, rotavirus strain characteristics revealed in sewage and contaminated surface basins were closely mirroring the strains normally responsible for the majority of infant disease cases [41, 49], but this may actually be true particularly for countries with lower sanitation where disposal of adult stools and children diapers is not kept separated.

A preliminary comparison between G- and P-types of rotavirus detected in either sewage samples or feces of children with diarrhea in Italy in 2010-2011 is reported in *Figures 1* and *2*, showing similar yet not identical distribution of viral strains.

Release and spread of animal rotaviruses may also result in contamination of surface waters, and food, favoring introduction of uncommon rotaviruses into human populations, that might ultimately endanger efficacy of current vaccines adopted for human use [12].

Due to the multiplicity of rotavirus strains possibly present in sewage or other environmental matrices at any time, genomic characterization of fundamental genes for origin attribution (*e.g.* VP7, VP4, and possibly NSP4, and VP6 encoding segments) may be of great value in monitoring the presence of emerging or uncommon rotavirus types circulating in a population, which are not yet but might subsequently get involved in symptomatic cases and epidemics. Similarly, environmental rotavirus genotyping may help identify the source of rotaviruses linked to epidemic outbreaks of disease [50]. However, due to the segmented nature of the genome it may not be possible to identify the whole genomic/antigenic formula of any rotavirus strains in sewage or water samples, possibly contaminated with multiple virus types deriving from an entire human or animal community [50, 51].

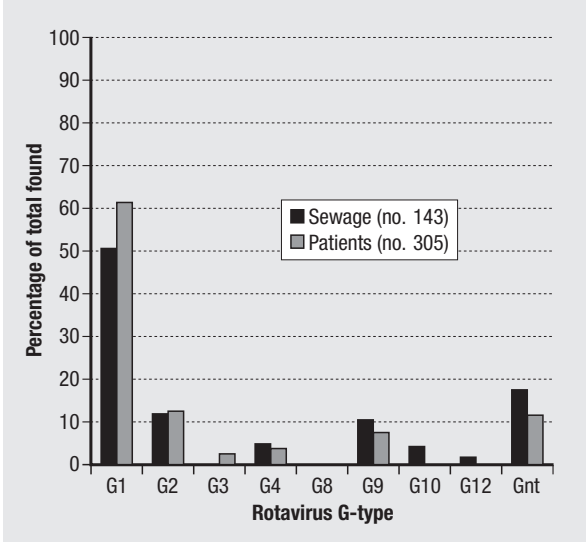

Fig. 1 | *Rotavirus G-types (percentage) detected in sewage (143 samples) or feces from children with diarrhea (305 samples), in Italy, 2010-2011. Nt = not typable.*

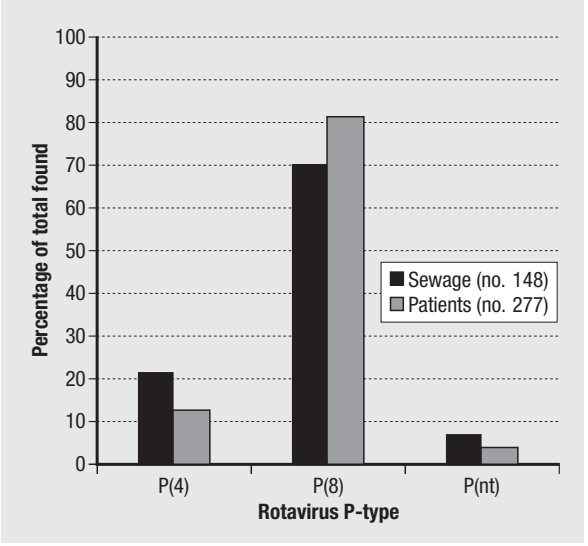

Fig. 2 | *Rotavirus P-types (percentage) detected in sewage (148 samples) or feces from children with diarrhea (277 samples), in Italy, 2010-2011. Nt = not typable.*

Rotavirus diagnostics in environmental samples

Whereas in sewage the concentration of rotavirus may be expected to be reasonably high, surface waters normally present low viral load [52], thus requiring extensive virus and RNA concentration protocols to be applied prior to detection and genotyping procedures by sensitive methods. These are treated in more detail elsewhere, and will only be discussed quickly in this section.

Protocols for concentration of rotavirus and other enteric viruses from sewage or clear water include adsorption-elution using negatively charged membranes, precipitation-flocculation, two-phase separation, centrifugation, tangential flow ultrafiltration, and gel chromatography.

Methods essentially similar to these can be successfully used also on vegetable rinsing extracts, optimizing virus detachment into the medium, or on sea mollusks.

Viability assay on rotaviruses present in naturally contaminated waters is impracticable due to low permissiveness of cell cultures to wild human strains, but viral RNA detection is more reliable .

Concerning molecular diagnostics and typing, a widely used protocol for feces analysis reported in the web site of the Rotavirus Surveillance Network "EuroRotaNet" (http://www.eurorota.net/), based on multiplex nested-PCR system following an RT phase with random primers, can be extended to environmental sample testing. For VP7 (G-genotyping), the protocol encompasses primers for common human rotavirus G-types G1-4, and G9, and emerging strains G8, G10, and G12. For VP4-typing, the multiplex assay covers the two common human P-types P[4] and P[8], and types P[6], and P[9-11] of possible animal origin. For special necessities, a full-genome typing approach may better help understand the evolution and occurrence of reassortment events of rotaviruses [14], although this is of hard applicability in the presence of multiple viral strains.

Rotavirus epidemic outbreaks

Although the children community faces winter-related major epidemics under temperate climate, rotavirus can also cause smaller epidemic outbreaks, involving all age groups but particularly children or elderly people, in schools, hospitals, nursing homes, and care centers, as well as grandparents in the household. Despite mortality, particularly in the case of elderly or subjects with reduced health conditions, the direct costs for the society related to outbreaks in long-term care institutions may be high, and long-term residence in a closed community is a risk factor for rotavirus illness.

Particularly in waterborne outbreaks, subjects of all ages can be affected presenting severe symptoms [53]. Increase of symptoms in adults in these cases is thought to be caused by the high virus load, which is often present in water sources contaminated with sewage, as seemed to be the case during a rotavirus outbreak associated with drinking water in Finland, characterized by particularly severe cases in both young and older children [54]. Although this outbreak was apparently caused by a common human G1P[8] rotavirus, in other epidemic cases of disease affecting elderly communities or older children it seems that less common genotypes may be involved, such as genotypes G2 and G4 [54].

Also because the issue of a differential immunity to viral serotypes is still unclear, other factors may explain these observations. It is reasonable to assume that the infectious rotavirus dose transmitted from person-to-person contact is likely small in countries with a high level of hygiene. On the contrary, larger amounts of virus might be present in drinking or surface waters contaminated by sewage spillover, and a high virus load might justify both severity of disease and involvement of subjects outside the normal age range observed in some outbreaks [54].

In fact, several studies suggest that both surface waters and some foodstuff can be massively contaminated with a multiplicity of viruses of human and/or animal origin, also including rotavirus, thus posing the conditions for triggering an outbreak [50, 54-56]. In some cases, it may be difficult to identify a unique etiological agent since different virus strains or even species may be detected in patients and environmental samples. As an example, apart from a single human strain only swine or bovine rotavirus-specific VP7 gene sequences were detected in four home tap-water drinking water samples during a rotavirus diarrhea epidemic affecting 56 children in France. However, all of these were different from the sequences detected in stools of patients [56]. This outbreak study also highlights the role of water pipelines as a vehicle of rotaviruses of both human and animal origin, possibly leading to co-infection of subjects with several rotavirus strains and consequent gene reassortment. As for other enteric viruses, similar conditions of risk may be present in filtering seafood, such as cockles, mussels and clams, which collect and may concentrate rotaviruses from contaminated waters, and are only lightly cooked or consumed raw [48, 57]. Rotavirus contamination of surface water may also end up in other food chains, including vegetables and soft fruit, correlated with irrigation. Although virus concentration may be lower on leaves and fruit skin, this type of food is a potential risk and has in fact been sporadically involved in outbreaks [32, 49]. Conditions for exceptional virus spread and large outbreaks may be generated by major natural events like flood and earthquakes [58].

Poliomyelitis. Virus, disease and vaccination

Poliovirus belongs to the genus Enterovirus within the family Picornaviridae, and strains are differentiated into three different serotypes. The genome is made of a single positive strand of RNA, which acts as a messenger RNA when released into the cytoplasm of susceptible cells, and is contained inside an icosahedral capsid made of 4 distinct proteins (VP1-4) [59].

The coding region of poliovirus genome is translated into a single polyprotein, which is then processed to generate the viral capsid (VP1 to VP4) and the nonstructural proteins. Surface-exposed loops in the capsid proteins VP1, VP2, and VP3 contain the antigenic determinants for poliovirus-neutralizing antibodies, and four main antigenic sites have been identified by the use of murine monoclonal antibodies [60].

Replication of poliovirus RNA is permitted by a virus encoded RNA polymerase with a high rate of error, which introduces frequent mutations in the genome at every replication cycle. Nucleotide substitutions may be selectively amplified during infections, ensuring an efficient mechanism for evolution of polioviruses through its progeny. Genetic recombination events between polioviruses and other clade C, non-polio enteroviruses (NPEVs) is another mechanism of evolution possibly leading to chimeric virus variants, exhibiting important phenotypic differences with respect to parental poliovirus [61].

Poliomyelitis is a very invalidating disease, although it develops in severe forms in a minority of

infected subjects. Consequently, vaccination has soon appeared as the only tool to prevent occasional emergence of severe paralytic cases by halting the wide circulation of wild neurotropic viruses throughout an asymptomatic population.

After ravaging the global population for thousands of years, eradication of poliomyelitis (WHO resolution WHA41.28, 1988) has thus been achieved in most part of the world, but a few areas remain with low endemic circulation of wild polioviruses sometimes generating epidemic outbreaks. The massive efforts towards global eradication has required vaccination of over two billion children during the past decades, and has resulted in a decrease from several hundreds of thousand to fewer than 2000 annual cases in 2010, to 156 in the first 9 months of 2012 *(Figure 3)*. Wild type 2 poliovirus was eradicated by 1999, and no further case due to this serotype has been reported thereafter. Most of the credit for breaking the endemic circulation of wild-type viruses worldwide goes to massive use of the Sabin's oral live attenuated polio vaccine (OPV), which was able to elicit an immune barrier against the virus at the intestinal mucosa level, resulting in a strong limitation for pathogenic poliovirus to replicate in the human gut and be shed with feces into the environment [62, 63].

However, disease has also been linked with infection with OPV vaccine-derived polioviruses (VDPVs), which can essentially arise by either person-to-person transmission among naïve, immune competent individuals (cVDPVs) or persistent infections of immune deficient individuals (iVDPVs) [64]. Infection with polio vaccine strains may quickly introduce substitutions that reverse neurovirulence attenuation into the progeny viruses. These viruses can evolve during replication in asymptomatic healthy persons or immunodeficient subjects, and give rise to vaccine-derived polioviruses (cVDPDVs), which exhibit characteristics of transmissibility and virulence similar to wild viruses. Therefore, establishment of OPV persistent infections in immune deficient individuals constitutes a threat for the unimmunized subjects and for the continuous shedding of potentially pathogenic virus, particularly when this is related to asymptomatic anonymous individuals. To avoid similar problems, some countries have always used the killed poliovirus vaccine originally implemented by Salk (inactivated polio vaccine, IPV) and derivatives, which is now largely used throughout the world alone or in a combined schedule with OPV, and is recommended after eradication [63]. The main disadvantages of the killed vaccine are its scarce induction of a mucosal immune response and need for parenteral administration, which are however counterbalanced by IPV inability to start any replication in the vaccine, and by its genetic and chemico-physical stability.

Eradication of polio and surveillance of poliovirus

With the ultimate perspective of global eradication of polio, the WHO has proceeded for decades throughout regional and local eradication programs, and large areas of the world are now established to be polio-free, including Europe since 2002 [65]. Clearance of new polio cases due to wild strains in a country must be confirmed for at least three consecutive years, and eradication status shall be sustained by continued mass vaccination with IPV and by an active surveillance plan of AFP cases within among the < 15 years old subjects, responding to specific requirements on the fraction of population surveyed. This is performed through a WHO-regulated network of national and sub-national, regional reference and specialized laboratories for diagnosis and surveillance, ensuring the epidemiological and laboratory investigation *(Figure 4)* of all cases of paralysis for poliovirus etiology, as an indicator of` poliovirus circulation (*Polio laboratory manual*, WHO/IVB/04.10). Additional measures encompass the identification, containment and destruction of all samples that might contain live wild or vaccine-

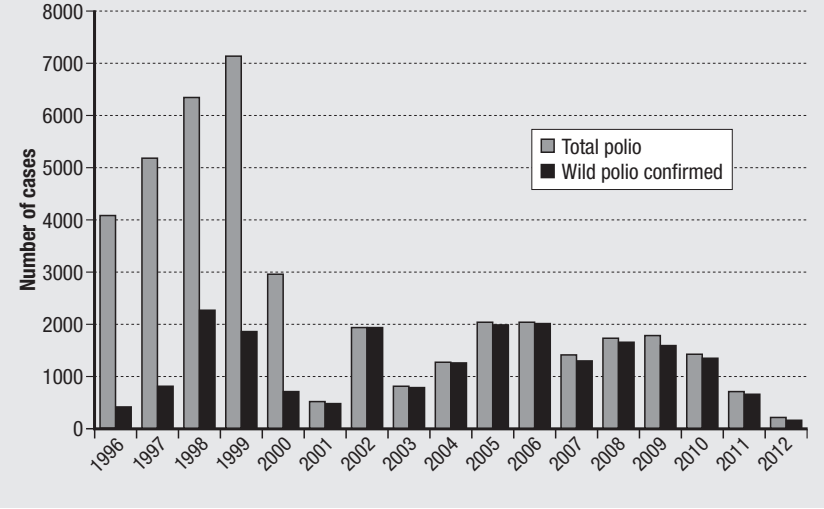

Fig. 3 | *Number of total poliovirus cases and confirmed wild-type polio in the world, 1996-2012. Graph is elaborated with data present in the WHO website (http://www.who.int/topics/poliomyelitis/en/).*

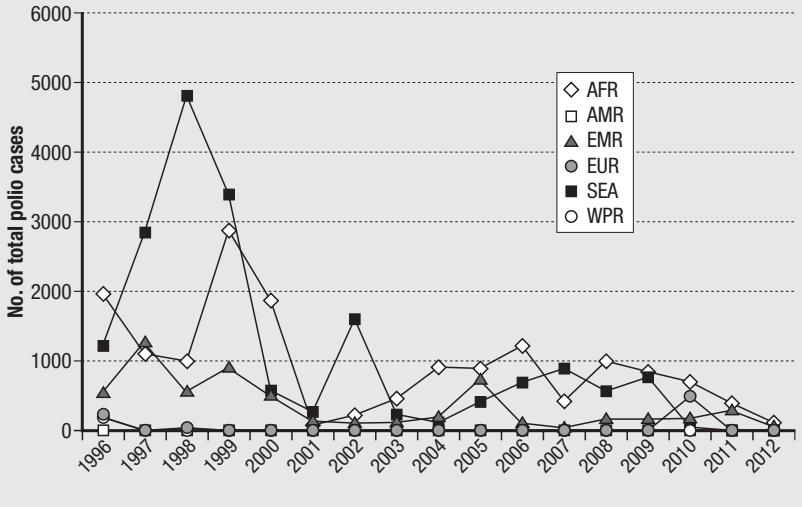

Fig. 4 | *Number of total poliovirus cases by geographic area, 1996-2012. AFR (Africa), AMR (Americas), EMR (Eastern Mediterranean), EUR (Europe), SEA (South-East Asia), WPR (Western Pacific). Graph is elaborated with data present in the WHO website (http://www.who.int/topics/poliomyelitis/en/).*

derived poliovirus or polio vaccine. In the case of endemic areas, universal vaccination of all children with OPV is still required in order to combat wild virus circulation.

As an example for European countries, Italy has adopted the mixed OPV-IPV schedule of vaccination in 1999, and a full IPV schedule in August 2002, maintaining approximately 96.5% coverage of complete vaccination in < 2 year-old children. The ongoing AFP surveillance program [66] indicates that Italy is polio-free, the last polio case due to indigenous wild virus having occurred in 1982 and the last imported wild polio case having come from Libya to receive specific medical care, in 1988. Because polio is no longer perceived as a frequent or emerging risk by the population and the physicians, AFP surveillance in Italy has requested more and more attention in order to fulfill the WHO performance indicators (*Polio laboratory manual*, WHO/IVB/04.10), whilst the geographical location of Italy, and the globalization and immigration dynamics have continued to put the country at increasing risk of importation of wild polioviruses or neurovirulent Sabin-derived polioviruses from areas with endemic poliomyelitis. The use of a full IPV vaccination has eliminated vaccine associated poliomyelitis cases (VAPP) in Italy, and has progressively decreased the circulation of Sabin poliovirus strains in both the population and the environment, but it has also limited passive immunization of contacts and the degree of herd immunity at the mucosal level.

It should be considered that the absence of clinical polio cases in a country does not necessarily imply the absence of poliovirus circulation. Acute flaccid paralysis (AFP) surveillance may thus conveniently be supplemented or replaced by enterovirus surveillance and/or environmental surveillance of sewage. Such supplementary surveillance, especially environmental surveillance, has proven in several instances to be a powerful tool for monitoring the importation and circulation of wild or vaccine-derived polioviruses before appearance of clinical cases, as well as for evaluating the effectiveness of control measures adopted in response [67].

The major obstacles to global polio eradication have been: a) the "failure to vaccinate" the susceptible cohorts particularly in countries characterized by poverty, war, and political or religious constraints; b) the "vaccination failures" in case of vaccine cold-chain breaches or immune depression linked to malnutrition; and c) the "emergence of VDPVs". Whereas availability of current optimized live attenuated or inactivated vaccines in endemic areas can help overcome the first obstacle, the other difficulties require an integrated worldwide approach, to reduce poverty and to monitor poliovirus circulation via immigration in the global world.

Environmental polio surveillance

As in the case of rotavirus and other viruses excreted with feces, also polioviruses can be spread in the environment and reach surface waters prompting environmental and food-borne transmission circuits, which in the end can return hazardous strains to susceptible humans. Besides being a risk source for poliovirus dissemination, sewage can be used to supply useful indicators of risk, independent of the current presence of diagnosed polio cases in the population.

Sewage surveillance is performed according to standard procedures recommended by the WHO (*Guidelines for environmental surveillance of poliovirus circulation*, WHO/V&B/03.03), and sometimes represents the only means to promptly identify introduction of wild or neurovirulent vaccine-derived polioviruses from endemic areas, particularly in in countries declared polio-free using the IPV. The increased immigration from areas with persistence of pathogenic poliovirus circulation is a present public health threat for Mediterranean countries such

as Italy but also for other developed countries in Europe and elsewhere, which can be monitored by environmental surveillance strategies. Also, environmental surveillance may permit to detect the presence of VDPV excretors in the community, even in the absence of clinical cases or outbreaks, a fact that may be ascribed to a level of herd immunity across the population sufficient to contain but not terminate transmission of neurovirulent polio. This implies that VDPVs shall be considered a potential source for outbreaks and for reemergence of polio even after eradication.

The distribution of VDPV excretors worldwide is in large part unknown, and the identification of asymptomatic shedders of VPDV in any specific area is a very difficult task. Countries with VDPV excretors are required to maintain a high herd immunity level by vaccine coverage, until shedding is extinguished. It shall also be considered that asymptomatic shedders might move from an area to another during their lives, changing the geographical risk conditions. If not recognized and properly contained, persistently infected VDPV excretors, particularly if immunodeficient, might shed poliovirus for years or even decades. Considering that the IPV vaccine is less effective than OPV at inducing mucosal immunity, possible circulation of poliovirus in IPV-immunized children might pass undetected despite active AFP surveillance is in place [65]. All of this makes the rationale for implementing environmental surveillance of sewage and surface waters in countries with risk of polio reintroduction.

Environmental polio surveillance virtually samples the entire population independent of cases of paralysis, as far as sampling sites are properly selected and investigation is performed using standardized methods (*Guidelines for environmental surveillance of poliovirus circulation*, WHO/V&B/03.03) [65]. With this in mind, a network of laboratories has also been built in Italy, using standardized sample collection methods on Italian wastewater treatment plants (WWTP), complementing the nationwide AFP surveillance activity, to gain evidence in support of the maintenance of polio-free status of Italy [66, 67]. No wild polioviruses were isolated from environmental samples after the surveillance started in seven cities in 2005, supporting the outcomes of AFP surveillance, but Sabin-like polioviruses were detected in sewage, although rarely and presenting a low mutation rate, in agreement with modest circulation of vaccine-derived strains in Italy. These findings are per se a confirmation that polio immunization is effective in Italy and generates high protection versus polio. However, since OPV vaccination was replaced with IPV in 2002, the polio Sabin-like strains found in the environment were most likely excreted by children immunized with the OPV in countries that still use Sabin vaccine, and shall thus be considered as imported cases from abroad, related to immigration flows. The possible risks of further amplification and genetic drift of these polioviruses especially in subjects with compromised immunity shall not be neglected. Surveillance on WWTPs yields relevant amounts of non-polio enteroviruses, which confirms the validity of the approach, but also requires detailed characterization to be performed on viral strains by both sensitive and specific molecular and cell culture methods (*Guidelines for environmental surveillance of poliovirus circulation*, WHO/V&B/03.03) [65-67].

Residual polioviruses in the 2000's

Risks associated with environmental spread of viral strains derived from the oral poliovirus vaccine (OPV) have emerged with particular high severity in the Dominican Republic and Haiti in the 2000's, where significant outbreaks of poliomyelitis have occurred among unvaccinated children [68]. A single type 1 vaccine-like strain was involved, presenting a highly mutated genome, that is suggestive of prolonged replication in the intestine of non-immune subjects [69], and neurovirulence and transmissibility characters similar to wild type poliovirus. These and other more recent findings on polio outbreaks in Africa and Asia [70] highlight the necessity that eradication of wild poliovirus from a country is promptly followed and sustained by adoption of vaccination strategies to prevent possible resurgence of the disease and continuing circulation of potentially pathogenic polioviruses.

The occurrence of sporadic outbreaks of poliomyelitis due to mutated vaccine-derived strains is also related to the presence of asymptomatic "long term excretors" of polioviruses in the healthy community. Studies performed on imunodeficient subjects in Europe (UK and Germany) and in US have revealed the presence of otherwise healthy subjects shedding vaccine-derived polioviruses presenting a marked rate of mutation toward the neurovirulent phenotype, who represent an actual risk of dissemination of highly pathogenic poliovirus strains in the environment and a threat for other unvaccinated or immunodeficient members of the same population.

Rotavirus and poliovirus vaccine strains in the environment, a problem or an asset?

During natural infection with wild pathogenic strains, rotavirus is shed at very high titers with human feces, often reaching 1010 infectious particles per gram lasting for several days or weeks [71]. This study was conducted on severely affected children, and highlights the chance for large amounts of infectious virus to be released into sewage, and possibly enter the environment and food routes of spread and transmission. Several other reports during decades of rotavirus investigation confirm this assumption, also using modern molecular detection approaches [72].

On the contrary, an early study with rotavirus vaccines for human use found limited vaccine rotavirus shedding by either vaccinated children or adults, but a possible effect of low level antibodies due

to previous unreported infection with wild virus could not be excluded. Conversely, studies following the introduction of either Rotarix or RotaTeq vaccines have confirmed measurable, although reduced, shedding of vaccine virus. Rotavirus antigen was detected in the stools of infants for more than a week after their first dose of pentavalent rotavirus vaccine [73]. A recent study in Australia, where the pentavalent RotaTeq is in use since 2007 [74], has shown some circulation of parental as well reassortant vaccine strains in both vaccinated children and subjects sampled in the community in the course of routine surveillance. Also, transmission of vaccine-derived rotavirus (RotaTeq) has been documented in a vaccinee sibling in US, resulting into symptomatic infection that the authors hypothesize may have involved reassortment with a co-infecting wild-type rotavirus [75]. Similarly, development of diarrhea appeared to be related to a double bovine-human reassortment occurred just after vaccine administration in 3 infants in Finland, concluding that this virulent vaccine-derived strain may have been release into the environment [76]

These and other reports are suggestive of an actual introduction of vaccine viruses, and vaccine-derived reassortants into the community, which may also involve environmental transmission.

Accidental ingestion of vaccine viruses via contacts with vaccinated children or contaminated fomites may pose a serious problem for immunocompromised subjects, where attenuated rotaviruses might start a serious symptomatic infection [77]. However, given the lower replication of last generation vaccine viruses compared to earlier vaccine strains or wild-type strains, this possible risk should not be taken as a reason for discouraging vaccination of susceptible cohorts.

After introduction of mass vaccination with the Rotarix vaccine in Brasil in 2006, environmental surveillance of rotavirus was performed on a major Wastewater Treatment Plant for along a year using RT-PCR and nucleotide sequencing [78]. Although all sewage samples examined were found to be positive for rotavirus, only wild-type genotypes were detected whereas no NSP4 or NSP3 sequences specific for vaccine-like strains were identified in any sewage sample. These data suggest that vaccine-derived rotavirus strains present in sewage are probably a minor part compared to the wild-type viral repertoire shed from the community as a consequence of natural infection. In different studies performed in Chile and Nicaragua after introduction of rotavirus vaccination, lower rates of rotavirus positive sewage samples were determined compared to other enteric viruses [79, 80], that suggests a possible reduction of cases of natural infection as a consequence of vaccine administration. For these reasons, environmental surveillance, particularly on wastewater, may represent an interesting approach to evaluate the potential impact of rotavirus vaccination on viral circulation in the community.

A final aspect concerns possible aids to susceptible children immunization via transmission of rotavirus vaccine strains excreted by vaccinees. A "passive vaccine passage" to unvaccinated contacts was considered a milestone in the fight to poliomyelitis using the live attenuated Sabin vaccines administered orally, but has lately been considered a risk of vaccine-related polio [69]. To date, several complete transmission dynamic studies on rotavirus vaccination have been published [81] exploring a variety of possible scenarios, but no study has approached the possibility of "passive contact immunization" for rotavirus vaccine. If any, this is however likely to be of minimal impact, considering both the reduced replication and shedding of vaccine strains by immunized subjects and the simultaneous co-circulation of more aggressive viral strains in the communities and the environment.

CONCLUSIONS

In conclusion, the environment and particularly surface waster can play an important role in transmission of rotavirus as in the case of other enteric viruses, not excluding polioviruses. Waterborne disease outbreaks or cases should be investigated by molecular characterization methods, in order to identify risk factors, possible spread of novel emerging viruses or reassortants, and apply control measures. Environmental surveillance on sewage treatment plants can help monitor shedding of uncommon viruses by a specific population, to promptly identify threats due to emerging viral strains in the community, and finally to assess ongoing vaccine programs since sewage screening may provide a rapid and economical overview of the circulating rotavirus genotypes. In the case of poliovirus, detection and characterization of polio and other enterovirus strains in environmental samples will supply more and more important information as the course towards global eradication progresses.

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Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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Impact of climate change on waterborne diseases

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Abstract. Change in climate and water cycle will challenge water availability but it will also increase the exposure to unsafe water. Floods, droughts, heavy storms, changes in rain pattern, increase of temperature and sea level, they all show an increasing trend worldwide and will affect biological, physical and chemical components of water through different paths thus enhancing the risk of waterborne diseases. This paper is intended, through reviewing the available literature, to highlight environmental changes and critical situations caused by floods, drought and warmer temperature that will lead to an increase of exposure to water related pathogens, chemical hazards and cyanotoxins. The final aim is provide knowledge-based elements for more focused adaptation measures.

Key words: climate change, waterborne diseases, microbial pathogens, chemical contaminants, toxic cyanobacteria.

Riassunto *(Effetto dei cambiamenti climatici sulle malattie trasmesse dall'acqua)*. I cambiamenti climatici e del ciclo idrologico metteranno a rischio la disponibilità d'acqua e aumenteranno l'esposizione ad acqua contaminata. Le alluvioni, le siccità, le grandi tempeste, le variazioni nella frequenza ed intensità delle piogge, il riscaldamento e l'aumento del livello del mare crescono in ogni parte del mondo e influenzeranno le caratteristiche biologiche e chimico-fisiche dell'acqua attraverso diversi meccanismi, con il conseguente aumento del rischio di malattie trasmesse dall'acqua. L'analisi della letteratura disponibile, presentata in questo articolo, evidenzia i cambiamenti ambientali e le situazioni critiche causate da alluvioni, siccità e crescente riscaldamento che causeranno un aumento di esposizione a patogeni, inquinanti chimici e cianotossine, legati all'acqua. Lo scopo è di fornire gli elementi scientifici di base per misure di adattamento mirate.

Parole chiave: cambiamenti climatici, malattie trasmesse dall'acqua, microrganismi patogeni, contaminanti chimici, cianobatteri tossici.

INTRODUCTION

Climate variability and change may greatly influence human health [1], directly, as for instance drowning or trauma in extreme weather events, or indirectly, by altering the characteristics of the natural environments and habitats hence increasing the exposure of human populations to risk factors.

Extreme weather events are by far among the most destructive disasters known, whether their toll is measured in lives, damages of built environment, destruction of critical infrastructure, loss of properties and economic activities, irreversible contaminations, forced population displacement, short and long term diseases. According to EM-DAT [2] disastrous weather events database (criteria to be accounted for EM-DAT data base: a) at least 10 people killed; b) more than 100 hundred people affected; c) call for international assistance; d) declaration of state of emergency), the number of affected people in the UN-ECE Euro Region in the last two decades has increased of about 400% compared to previous decadal period. Up to the first semester of 2008, as a consequence of adverse meteorological disasters, 38 million people required health assistance and basic survival needs such as safe shelter, medical assistance, a safe water supply and sanitation. EU accounted for 29 million of affected people with an economic loss of about 270 US$ billion, the highest rate in the world of economic loss per capita. In particular, Italy and Germany suffered major damages from floods and storms due to high population and infrastructure density. The overall scenario in the western hemisphere is similar. In the US more than 700 billion US$ in damages were estimated for the period 1980-2008, mostly due to hurricane, severe weather and non-tropical floods. Trends constantly increased worldwide and they're expected to do the same in the future since, as stated in the latest 2012 IPCC SREX Report [3], observed changes in climate extremes reflect the influence of anthropogenic climate changes in addition to natural climate variability, with changes in exposure and vulnerability influenced by both climatic and no climatic factors. Specifically about floods, it is

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likely that the frequency of heavy precipitations or the proportion of total rainfall from heavy rainfalls will increase in the 21st century over many areas of the globe. This is particularly the case in the high latitudes and tropical regions, and in winter in the northern mid-latitudes. Heavy rainfalls associated with tropical cyclones are likely to increase with continued warming induced by enhanced greenhouse gas concentrations. There is high confidence that locations currently experiencing adverse impacts such as coastal erosion and inundation will continue to do so in the future due to increasing sea levels, all other contributing factors being equal [3].

In the temperate zone, climate change is predicted to decrease the number of rainy days, but to increase the average volume of each rainfall event [4]: as a consequence, drought-rewetting cycles may impact water quality as it enhances decomposition and flushing of organic matter into streams [5]. Flooding is the most common natural extreme weather event in the European Region [2]. Flooding may be caused by heavy rainfall, tidal surges and rapid snow melt. According to a database on floods in Europe, the most extreme flash floods are greater in magnitude in the Mediterranean countries than in the inner continental countries [6]. Coastal flooding related to increasing frequencies and intensities of storms and Sea Level Rise (SLR) is likely to threaten up to 1.6 million additional people annually in the EU alone [7]. Larger storm surges produced by extreme storms, combined with a rising sea level, could result in much higher rates of coastal erosion, which would in turn affect the levels of saline intrusion into coastal freshwater [8].

Other climatic factors affecting the hydrological regime are temperature and droughts, both of them projected to become worse.

About temperature, models predict with large confidence a substantial warming in temperature extremes by the end of the 21st century. Also, on a global scale, the frequency and magnitude of warm daily temperature extremes will increase, while cold extremes will decrease. The frequency of annual hottest days are projected to go from 1 in 20 years to 1 in 2 years, with an increase in the annual daily temperature of 2 to 5 °C by the end of 21st century, even if regional variations will often differ from the global changes [3].

These phenomena are going to affect many characteristics of water basins, as it has already happened. Atmospheric warming has been associated with an increase in surface water temperatures since the 1960s in Europe, North America and Asia (0.2-2 °C) [4]. In several lakes in Europe and Northern America, the water temperature increase has influenced the stratification period that has lengthened by 2-3 weeks [9]. In the European rivers Rhine and Meuse, an increase in the average summer water temperature of about 2 °C has been observed over the last three decades, with temperature peaks during the two severe droughts in 1976 and 2003, with a pH increase (due to a decrease in CO_2 concentration) [10-11]. Computer models predict an increase of around 2 °C by 2070 in European lakes, although differences can be estimated, depending on lake characteristics and season [12-13]. The residence time in lakes with, at present, a short residence time, will probably increase by 92% in 2050 in summer and there will be a significant increase in temperature in the epilimnion and hypolimnion in shallow lakes [13]; however, on a long period, deepest lakes will be most sensitive to warming, due to their higher heat storage capacity and will experience highest winter temperature [14].

Also the ocean state has changed, in response to changed surface thermal conditions. The heat content of the World Ocean has increased since 1955, leading to sea level rise through thermal expansion, in addition to transfer of mass from glaciers, ice sheets and river runoff, due to changing hydrological regime. The waters at high latitudes (poleward of 50°N and 70°S) are fresher in the upper 500 m, while the subtropical latitudes in both hemispheres are characterized by increase in salinity. However, while there are many robust findings regarding the changed ocean state, key uncertainties still remain, making difficult projections for the future [15].

Droughts, which can be described as an unusual long period with little or no precipitation, are expected to increase in some areas. Model projections for the next 50-100 years indicate that climate change will reduce discharges to coastal waters in southern South America, western Australia, western and southern Africa, and in the Mediterranean Basin with consequences on salinities and nutrients and sediment delivered to the coast [16]. Salinity will tend to advance upstream, thereby altering the zonation of plant and animal species as well as the availability of freshwater for human use. Saltwater intrusion as a result of a combination of sea-level rise, decreases in river flows, unsustainable freshwater withdrawal and increased drought frequency are expected to modify physical and chemical components of estuarine-coastal environments with secondary impacts on phytoplankton community. In Central and Southern Europe and the Mediterranean region, in North and Central America, northeast Brazil and Southern Africa, droughts will likely intensify in the 21st century, due to reduced precipitation and/or increased evapotranspiration [3]. Projection for the rest of the world are still inconsistent, due to lack of data, or incapacity of the model to include all the different causes of dryness [3].

During dry periods, reduced groundwater recharge and increased water abstraction due to warmer temperatures may cause further water stress by reducing groundwater table levels. For these reasons in coastal areas, droughts can cause the intrusion of seawater into freshwater aquifers. In general freshwater contamination by seawater of only 5% is enough to rule out many important uses including drinking-water supply, irrigation of crops, parks and gardens, and

the well-being of groundwater- dependent ecosystems [17].

To identify the role of climate change on the spreading of waterborne diseases is made difficult by the simultaneous influence of other causes, like destruction of habitats, extensive travels and migrations of human populations, drug and pesticide resistance, urbanization and increased population density, and availability of health services [18].

The aim of this paper is to review the available literature to show the potential increase of the burden of WBDs resulting from climate changes and particularly from floods, increase of temperature and droughts, with regards to risk factors as microbial pathogens, chemicals, cyanotoxins.

MICROBIAL PATHOGENS

Waterborne pathogens of human and animal faecal origin include a high number of viruses, bacteria and parasitic protozoa. Also several naturally occurring microorganisms can be pathogenic to humans, as various species of *Vibrio* (gastroenteritis, diahorraea and septicemia), *Pseudomonas aeruginosa* (skin and ear infections), *Legionella pneumophila* (Legionnaire's disease) and amoebae (encephalitis) [19].

Waterborne pathogens of concern for humans have the following characteristics:

- "are shed into the environment in high numbers, or are highly infectious to humans or animals at low doses" (*i.e.* cystis of protozoa);
- "can survive and remain infectious in the environment for long periods, or they are highly resistant to water treatment;
- some types of bacterial pathogens can multiply outside of a host under favorable environmental conditions" [20]. Pathogenic microorganisms of human and animal faecal origin enter surface waters mainly by discharges of raw and treated wastewater and by runoff from the land. The reservoirs and routes of exposition are schematically depicted in *Figure 1*, after Hurst [21]. Some pathogens, like *V. cholerae*, hepatitis A virus and *Schistosoma* are restricted to tropical areas, while others like *Cryptosporidium* and *Campylobacter* are more diffused [22].

Heavy rainfall and floods

More frequent and intense heavy rainfall/floods will cause higher pathogen concentrations in natural waters which will generally be reflected in worse quality of drinking and bathing waters, crops and shellfish. Indeed, heavy rainfall/floods can cause over-flooding of sewage treatment plants, runoff of animal dejections and manure, re-mobilisation and redistribution of contaminated sediments [7, 23-27]. Since the diffusion of pathogens depends on hydrodynamic of surface water bodies it can be expected that floods and heavy rainfall, by speeding up water fluxes carrying pathogens, will counteract the natural pathogen inactivation in the environment by UV and temperature.

Enhanced environmental levels of pathogenic microorganisms may result in increased incidence of diseases and occurrence of new ones [28]. In general it is expected that zoonotic infections may expand due to an increased washing into water of wild animal and livestock faeces. A significant problem can be posed by an increased presence of different strains of enteric viruses in water bodies, as they are resistant to treatment in sewage treatment plants; bathing water receiving treated waters and seafood reared in receiving water bodies can represent important source of exposure to these pathogens [29]. Furthermore, viruses can be the unseen etiological pathogens responsible for human diseases even

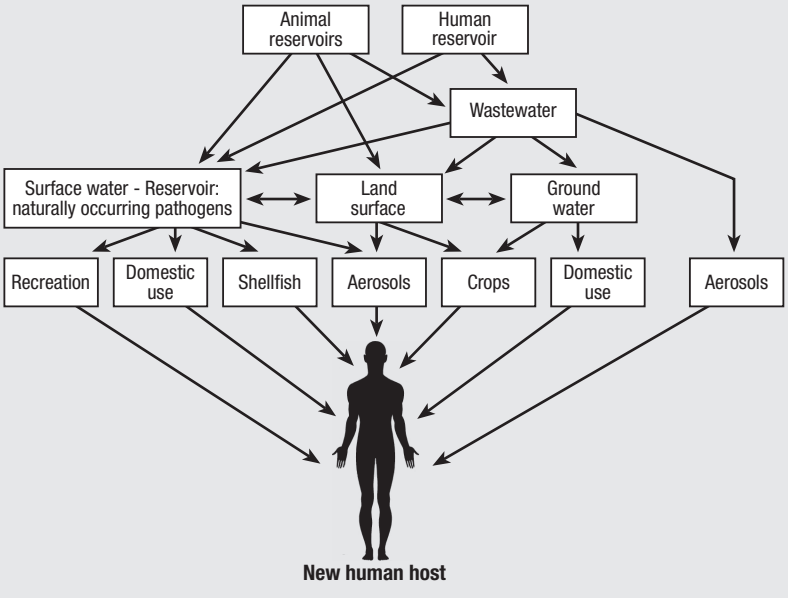

Fig. 1 | *Sources of waterborne pathogen. The non-enteric pathogens are naturally present in surface waters, which can, therefore, be a reservoir. (Modified from Hurst [21]).*

when waters meet regulatory criteria for faecal contamination, based on conventional bacterial indicators, which are less resistant than viruses and decay much faster in natural environment [29]. Studies on cryptosporidiosis suggest that in the future more intense precipitation events may increase the saturation of soil profiles and mobilize infectious oocysts more often, significantly increasing the risk [30].

There are several examples of waterborne diseases outbreaks associated to excessive rainfall [31-43]. The largest reported waterborne disease outbreak in the United States, due to the presence of *Cryptosporidium* cists in drinking water, occurred in Milwaukee in 1993 and was related to heavy rainfall and associated runoff and consequent contamination of Milwaukee lake, the source of the waterworks of the area. It resulted in the deaths of 54 people and more than 403 000 ill [37, 44]. Contamination of groundwater after flooding has been associated to additional disease outbreaks like *Acanthamoeba keratitis* in Iowa (USA) [22]. An outbreak of giardiasis in Montana (USA) was related to excess rainfall [39]. *Cryptosporidiosis* cases in England and Wales were positively associated with maximum river flow [45]. *Escherichia coli* O157:H7 and *Campylobacter jejuni* were responsible for a waterborne outbreak, causing 7 deaths, 65 hospitalization and more than 2300 cases of gastrointestinal illness in the Canadian town of Walkerton [46]. In this case, drinking water, supplied by shallow groundwater wells, turned out to be contaminated by a cattle manure from a local farm, following a period of intense spring rainfall, an event that is considered to happen once every 60 years [46].

In the US, Curriero *et al.* [38] reported a statistically significant association between excess rainfall and waterborne disease outbreaks over a long period of time and on a national scale. The study was based on 548 reported outbreaks in the United States from 1948 through 1994. The results indicated that 51% of waterborne disease outbreaks were preceded by rainfall events above the 90th percentile and that 68% were preceded by events above the 80th percentile. Outbreaks due to groundwater contamination were preceded by a 2-mo lag in rainfall accumulations whereas surface-water contamination showed the strongest association with excessive rainfall during the month of the outbreak [38].

In the European Union, in 2007, only 17 waterborne outbreaks were reported by eight countries, clearly indicating an under-reporting; they involved 10 912 cases, with 232 hospitalizations. The main microorganisms involved were *Campylobacter*, norovirus, *Giardia* and *Cyptosporidium* [47]. Only 24% of waterborne pathogen outbreaks in England, between 1970 and 2000, were found associated to heavy rainfall [48]. Yet, Nichols *et al.* [36] analyzing a small dataset [89] of waterborne outbreaks in England and Wales between 1910-1999, due to *Giardia*, *Cryptosporidium*, *E. coli*, *S. typhi*, *S. paratyphi*, *Campylobacter* and *Streptobacillus moniliformis*, assessed a significant strong correlation between 40% of the cases and heavy rainfall in the week before the outbreak or low rainfall during the four weeks preceding the outbreaks.

Floods and hurricanes, destroying the water distribution system and mixing drinking and waste waters, can have a significant impact also on the diffusion of cholera, caused by the naturally occurring *V. cholerae*. The disease is one of the most severe forms of waterborne diarrheal disease, especially for developing countries, where outbreaks occur seasonally and are associated with poverty and use of poor sanitation and unsafe water [1]. The extreme climatic events increase the cases of diseases and fatalities by adding an oral-faecal contamination pathway difficult to manage.

Rainfalls are known to worsen the microbiological quality of bathing waters, indeed they are used directly as predictors of short term events of contamination in the European directive concerning the management of bathing water quality [49]. The same approach should be applied to shellfish-growing waters, as well. Indeed after heavy rainfalls sudden contamination of coastal waters are expected and results from environmental investigation, which require a too long time, do not represent the adequate tool to predict this contamination and prevent from dangerous human exposures. Heavy rainfall and sewage treatment plant failure were twice responsible for international gastroenteritis outbreaks due to consumption of oysters harvested from Tahu lagoon, in France [50, 51]. One of the outbreaks was characterized by the high diversity of human enteric viruses (up to six different strains) detected both in patient stool and shellfish samples [51]. Other important outbreaks associated to contaminated oyster/clam consumption caused by sewage overflow and discharge into the aquatic environment during heavy rainfall events were reported as those that affected 2000 people in Australia, in summer 1978 [52], and 1000 people in New York State in 1982 [53].

Temperature

There is a contrast between the well documented and forecasted increase in temperature and the paucity of data on the effects of this increment on microbial pathogens and infectious diseases [54].

Enteric pathogens in the water environment are generally neutralized by higher temperatures, however, their sensitivity shows different features. For instance, cysts of *Giardia* and enteroviruses are less rapidly inactivated compared with oocysts of *Cryptosporidium* [55]. *Cryptosporidium* oocysts are inactivated during the winter, as they are susceptible to freezing and thawing cycles; hence, as the number of frozen days decrease, oocysts may increasingly survive through the winter [30]. It is also known that a large variation in temperature susceptibility exists among viruses [56], suggesting a possible selection of more resistance strains, like the hepatitis A virus (HAV), which is fairly temperature insensitive. For some viruses a

correlation between increasing temperature and inactivation rate starts only after 10 days [55].

Increasing temperature could favor less temperature sensitive species, directly promoting the growth of some indigenous bacteria, including pathogenic species [57]. Vibriosis caused mainly by *Vibrio parahaemolyticus, V. vulnificus* and *V. alginolyticus* are among the 6 most common foodborne diseases monitored by the Foodborne Diseases Active Surveillance Network (FoodNet) in the USA, where *V. parahaemolyticus* in the past decade has become the main cause of gastroenteritis [58]. According to CDC, the incidence of *Vibrio* infections in the USA, which are also monitored by the Cholera and Other *Vibrio* Illness Surveillance (COVIS) system and are due to exposure to recreational water as well, was 76% higher in 2011 than in 1996-1998 [59]. A strong link between rising summer water temperatures, prolonged summer seasons, and noncholera *Vibrio* sp. infections has been shown [60], even if Johnson *et al.* [61] found that temperature explain only about 50% of the presence of *V. paraemolytichus*, and also that the pathogenic subpopulations respond differently than the whole population to temperature.

The influence of increased temperatures on environmental bacteria is not expected to be homogeneous. The Baltic Sea for instance provides an environment in which only small changes of the present conditions (*e.g.*, temperature) result in increased *Vibrio* sp. populations [60].

Increasing temperatures would be expected to expand the range and increase the prevalence of *V. cholerae* and cholera both geographically and temporally, if environment and public health measures are not implemented [57]. Indeed, temperature shifts will alter the latitudinal distribution of planktonic species. Due to sea level rise inland areas will experience greater saltwater intrusion and increased levels of marine and estuarine bacteria, including *V. cholerae* [57]. It is then expected that an increase in temperature will threat water quality with regard especially to cholera disease in Asia and South America [22]. However, the question may be more complex. A non linear population model to explain and predict the dynamics of *V. cholerae* has been developed by Koelle *et al.* [62]. The authors, considering both extrinsic (climatic) and intrinsic (acquired temporary immunity) drivers of epidemics, could explain the interannual cycles of cholera outbreaks from Matlab, Bangladesh. They found a strong correlation with climatic variables (monsoon) over 7 years and with local water temperature, degree of floods and droughts, at a shorter scale. Warmer pond and rivers increase the incidence of cholera through the faster growth rate of the pathogen in aquatic environments, but to have a good correspondance between the model and the incidence of cholera epidemics it was necessary to include in the model data on other parameters included the complex dynamics of aquired temporary immunity which could explain the interval between two epidemics [62].

There are also some authors who argued that one of the main effects of climate change is the reduction of biodiversity, that is some species are disappearing at a high rate, and that pathogens are subjected to the same ecological constraints [63]. Therefore, if there are areas where health conditions are worsening due to the spreading of pathogenic organisms, there are other areas that will be affected in the opposite way, thus reducing the areal distribution of the same pathogen. The final balance could be no overall increase in the global diffusion of a pathogenic species. The importance of such a debate is to highlight, among others, some important aspects to be considered: 1) better data sets and modeling approaches are required to make robust predictions of the impacts of climate change on disease dynamics and 2) expansion or reduction of specific pathogens geographical ranges will depend not only on extrinsic factors (including climate change), but also on intrinsic factors (such as immunity, phenotypic plasticity, and evolution) [64].

Droughts

The impacts of drought on human health due to shortage of water are dramatic and include deaths, malnutrition, increase in infectious diseases [65]. These effects are associated with worsening hygienic conditions, higher probability of microbial contamination of drinking water due to infiltration of organic material along the distribution system when pressure drops, higher re-use of wastewaters in agriculture, with consequent contamination of fresh vegetables, unsafe use of untreated water. Additionally, water shortages may increase the likelihood of multiple uses of a water body (*e.g.*, for cleaning, bathing, and drinking) with a consequent increase of the risk of microbial contamination and human exposure to pathogens [57]. Periods of droughts followed by short intense rainfall can cause peaks of surface water contamination. River-bed sediments represent an important microorganisms reservoir in dry areas, like the Mediterranean, where long dry periods are interrupted by flash-floods, transporting most of them downstream, up to coastal waters [66]. In areas affected by increasing droughts, treated effluents from sewage plants might become a quantitatively important source of water influx into river and heavy rainfall events can pose a risk to human health because of the huge increase of pathogenic microrganisms concentrations in the river downstream sewage treatment plants. In a study on *Campylobacter*, it has been shown that the resulting combined sewer overflow, enriched in pathogen, is discharged into the receiving rivers at a 150 fold higher concentration than usual [67].

Drought can lower the water table, resulting in changes in underground water flows of surface water into groundwater. In UK, an outbreak of *Cryptosporidium*, due to contamination of the borehole used as a source of drinking water was recorded when unusually very strong rainfall followed a long dry period [68]. No conclusive explanation is

given in the report, but the authors suggested the possible contamination of the aquifer by the intrusion of a contaminated river water flowing nearby, through interstices in the chalk [68]. Other examples of outbreaks linked to contamination of groundwater sources have been documented. In Brushy Creek, Texas, an outbreak of cryptosporidiosis was reported in 1998 followed extended drought conditions. The primary drinking water supply of Brushy Creek was chlorinated groundwater [69]. This source was contaminated by sewage, through fractures in the bedrock due to the long period of drought and extreme heat in which heavy water demand and no rainfall was present to recharge the aquifer [69]. Outbreaks of cryptosporidiosis have been associated to contamination of drinking water supplied by surface water, due to intense rainfall after very long and unusual drought periods, in Japan and Oregon [70-71]. The largest reported outbreak of *E. coli* O157:H7 occurred at a fairground in the state of New York in September 1999 and was linked to contaminated well water. This outbreak resulted from unusually heavy rainfall, which was preceded by a drought [43].

In general, monitoring programs are not elaborated in such a way to capture these periods of very high peaks of pathogenic concentrations which in turn represent a particularly high risk of infectious diseases transmission.

CHEMICALS

Climate change may influence the concentrations of chemical constituents and contaminants in natural waters through diverse ways. As an example, coastal erosion, likely to be exacerbated under climate change, has led to the exposure of landfill sites in Europe, with a clear potential for contamination of coastal waters [27].

It is difficult to estimate the risk associated to increased chemical concentrations in natural waters. Nevertheless, it seems possible to identify the following scenario of human exposure and the respective reasons of concern.

Heavy rainfall and floods

Climate change will influence the concentration of chemical constituents in natural waters. Indeed, it has been reported that a storm flow, by increasing the concentration of dissolved organic matter to which some metals can strongly get complexed, could lead to a transport of dissolved lead, titan and vanadium in peat land systems [72]. Furthermore a seasonal change in dissolved metal concentrations has also been observed for various trace elements (Fe, Mn, Al, La, U, Th, Cd and As). An increase of organic carbon content and a decline in redox conditions seem to be related with a trace elements release. A positive correlation is also found between storm events and trace element concentrations in streams [73]. In fact, organic and inorganic colloids could play an important role in trace elements mobilization in soil sand water [74]. The possible implications for human health associated with these higher mobilization of chemical constituents from minerals and higher concentrations in natural waters are difficult to demonstrate. Natural waters contain many minerals, often in very small concentrations, that arise from the contact with the rocks and soils that water goes through. Most of these are of no concern but some are known they may impact human health for instance via drinking water, as arsenic and fluoride. Both arsenic and fluoride are significant contributors to morbidity in regions where concentrations in water are high. Hence, higher concentration of these elements as well as others of human concern may represent higher risky conditions in the affected areas.

More frequent intense rainfall is likely to exacerbate the flushing to water bodies of agricultural pollutants, including pesticides and veterinary medicines [28, 75]. Flooding may lead to contamination of water other than with chemicals already in the environment like pesticides, with dangerous chemicals, heavy metals, or other hazardous substances, from storage. Overall, flood events and strong rainfalls can transport pollutants from a contaminated area to a non-contaminated one [24, 76], from soil and sediments to water bodies.

Drinking water treatment is vital in protecting public health against microbial illness which remains a major cause of morbidity and mortality in many parts of the world, yet care has to be taken that treatment does not introduce higher levels of unwanted disinfection by-products (DBPs) than necessary. Indeed, epidemiological studies reported weak associations between chlorination and cancers of the colon, rectum and bladder and positive associations between concentrations of DBPs and a number of adverse reproductive effects, particularly stillbirth and low weight for gestational age [77]. In the past years a significant decline in DBPs concentrations has been achieved especially thanks to the introduction of improved treatment to remove the natural organic matter and better filtration that means less chlorine to be added. Floods and heavy rainfalls may increase concentrations of organic precursors of DBPs in surface waters [14]. On the other hand they cause higher nutrient concentrations which can promote algal and cyanobacterial blooms. Chen *et al.* [78] showed that DBPs precursors originating from high cyanobacterial densities can account for significant percentages of the total DBPs formation potential.

Increased floods and rainfalls will alter the transport, transfer, deposition and fate of chemical contaminants in coastal waters. Bioavailability of specific contaminants (*e.g.* metals) is greatly affected by salinity [79, 80]. Numerous studies have shown an increasing metal uptake by diverse aquatic organisms at reduced salinities [81-83]. Other studies have shown that solubility of many PAHs depends on salinity in the ambient water [84]. Thus, floods and

rainfalls will regulate the extent of exposure to toxic substances of seafood, hence of its consumers.

Unfortunately there is still little published evidence demonstrating a causal effect of chemical contamination on the pattern of morbidity following flooding events. But it is expected that floodings and heavy rainfalls, having implications for residue levels in food crops, food animals [85] and water bodies [86], will increase human exposure to chemical contaminants.

Temperature

It is well known that temperature influences physic chemical equilibriums and biological reactions. Chemical reactions can be doubled for a temperature increase of 10 °C. As a consequence, higher water temperature will determine an increase of dissolution, solubilization, complexation, degradation, evaporation. Hence higher temperatures will lead to higher concentration of dissolved substances in water but will also favor an increase of volatilization of chemicals.

Considering the specific issue of pesticides, higher temperature will exert contrasting impacts, from one side higher degradation and volatilization [75] − whch often will cause a more intensive use of them to fight pests risk − but from the other side, more solubilization in water. It is likely that changes in land use, induced by higher temperature, might play a more significant effect on pesticides in the environment than transformation and repartition processes [75]. For example, an increased prevalence of pests, weeds and diseases may lead to wider and more frequent application of both pesticides and veterinary medicines [28, 75].

Higher temperature in the Oceans may increase human exposure to mercury, especially in geographical areas where the population diet is based on seafood. Mercury is a global pollutant and is a reason of concern for public health when it is elevated above natural background levels, mainly through anthropogenic causes [87]. Mercury seems methylated by biotic processes [88]. Monomethyl mercury bioaccumulates and biomagnifies at all trophic levels in the food chain and can have severe neurological effects. Mercury methylation rates are temperature dependent [89]. Usually 80-99% of mercury found in fish muscle tissue is methyl mercury, regardless of its concentration in the environment [89]. It is expected that ocean temperature changes will increase the number of people exposed to above the tolerable weekly intakes defined by WHO for mercury [90].

Increased temperatures of natural surface waters used as drinking water supplies promote the DBPs formation rate [14]. Rodriguez and Serodes [91] showed that thrialomethanes (THM) concentrations vary from 1.5 to 2 times, depending on the utility, between drinking water plant and tap. In the same way, others authors reported that increasing temperature (10-33 °C) generally increased the formation of bromoorganic DBPs [92].

Droughts

Hotter, drier summers and increasingly severe and frequent droughts will deplete river flows, reducing contaminant dilution capacity and leading to elevated concentrations of hazardous substances [27]. Dry periods may entail brief spikes of compounds from sewage effluents, which can provoke transient perturbation of river ecosystems [93], with possible implications for human health. Increased contaminant concentrations are expected also in groundwater aquifers, especially in the unconfined ones.

Connected to water shortage is the particular case of temporary rivers. These are characterized by periodic dry phase and are a significant percentage of total river length in the world (between 40 and 70%). They are dominant in the semiarid Mediterranean area [94], where they represent an important source of water. These ecosystems are particularly exposed to the alteration of hydrological cycle due to longer drought periods associated with intense runoff and flushing [16], which can bring particularly high concentrations of pathogenic microorganisms and chemicals. The rising interest in their ecology during the last decade has highlighted their importance as links between water stored in soils, aquifers, snowpack, glaciers, vegetation and the atmosphere [95]. Recently, it has also been shown that they are important spots of nutrient and carbon recycling during the dry period [96, 97]. These processes are strictly related to the biochemistry of hazardous substances, entered the river along the hydrographic basin. Increasing frequency and duration of alternated dry-heavy rain periods, due to climate changes, by altering the timing of these processes is definitely going to affect the impact on the coastal environments of the catchments. Whether in a positive or negative way, there are not enough data yet [98].

CYANOBACTERIA

Health risks due to the toxins produced by cyanobacteria, photosynthetic prokaryotes diffuse in all the habitats, especially the aquatic ones, have been extensively addressed in this same issue of *Annali dell'Istituto Superiore di Sanità* by Manganelli *et al.,* p. 415 [99]. Briefly, cyanotoxins have different toxicological profiles, and target different organs. Several environmental factors affect their production, but mechanisms are still unknown. Humans can be exposed to cyanotoxins via ingestion (drinking and bathing water, through aquatic food chain), via aerosol and via parenteral, if surface contaminated water is used for haemodialysis [99]. Climate changes effects will be summed up with the effects of other environmental variables on cyanobacterial fitness and toxicity, with different outcomes, depending on the species/strain and the environment (*i.e.* lake, river, seawater). Available data are mostly obtained in laboratory studies or using mesocosm systems, even providing conflicting results: in many cases the apparent discrepancy could be attributed to the diverse response to climate change of differ-

ent cyanobacterial species, strongly depending on their physiological or ecological features, or, in the case of field studies, to the different response of the water body. It is therefore not possible to draw some general behaviours for the entire class of cyanobacteria and separated analyses should be carried out depending on the species and the environment. Bearing in mind these considerations, it is possible, at present, only summarize the available information in the following as shown in *Figure 2*.

Heavy rainfall and floods

Changes in freshwater runoff will have the greatest potential impacts on estuaries, causing changes in physical mixing characteristics [100]. Freshwater inflows into estuaries influence water residence time, nutrient delivery, vertical stratification, salinity, and control of phytoplankton growth rates [101]. Increased freshwaters inputs from increased runoff can dilute the estuarine environment by lowering salinity and promote extremely large blooms of toxic cyanobacteria by carrying *inocula* of salt-tolerant cyanobacteria from inland rivers. A combination of a particularly strong freshwater inflow after one day of unusual heavy rainfall with high temperature and the right nutrient concentration, favoring fast growth rate, has been the cause for a very large bloom of *Microcystis aeruginosa* in the Swan River Estuary, West Australia, in February 2000 [102].

Since increasing salinities can induce cell lyses and/or extracellular release of toxins [103], an indirect effect of heavy rainfall/floods, by conveying toxic cyanobacteria to brackish coastal waters, can be an increase in the extracellular quota of toxins. In 2005 in St. Lucie River Estuary, one of the largest brackish water systems on the East Coast of Florida, a periods of heavy rainfall caused the washout of *M. aeruginosa* cells into the estuary, suddenly covered by a dense bloom: the abrupt change in salinity (32‰) caused an increase of 80% of toxin release in water [104]. Heavy rainfall and floods enhance the possibility of cyanobacteria expansion into coastal environments, thus increasing the exposure of edible organisms [99]. It is worth noting that heavy rain and floods can increase the nutrient availability of lakes, that in turn induce cyanobacterial proliferation. In Bangladesh, where a heavy rainfall drained phosphorus from the surrounding paddy fields into an aquaculture ponds, the increased nutrient concentration (9.5 mg L^{-1}) coincident with high temperature (31 °C) was the possible cause of a bloom of *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* [105]. Soil runoff and discharge from wastewater treatment plants, rich in phosphorus, iron and carbon, might be responsible for the spreading of the mat-forming nitrogen-fixing *Lyngbya maiuscula* in coastal Queensland [106-107]. In Denmark the increase in phosphorus loading from land to lakes and coastal areas is expected to increase by 3 to 16% in the next 100 yr, due to higher winter rainfall; a shift in lakes community has already been observed towards dominance of cyanobacteria and dinoflagellates *vs*. diatoms and crysophytes [108].

Temperature

Increasing temperature in eutrophic condition can directly increase cyanobacterial proliferation and can be a main factor for their poleward movements and possibly their toxicity [109]. Indeed, some cyanobacteria like *Microcystis*, *Anabaena* and *Oscillatoria* have higher growth rate at higher temperature with respect to diatoms [110-112] and temperature can be one of the main factor in determining the success of one species *vs* other cyanobacteria or other phyto-

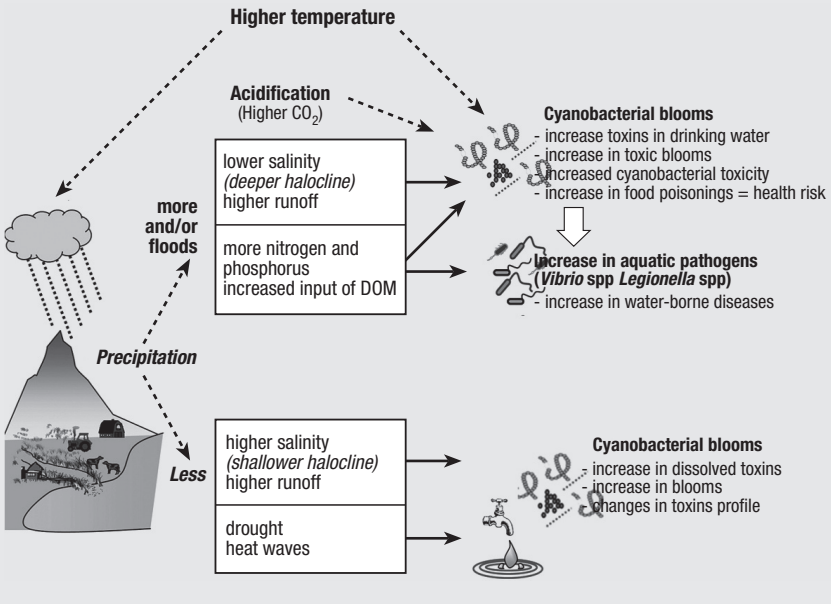

Fig. 2 | *Schematic diagram depicting the interrelations between changing meteorological parameters and toxic cyanobacteria.*

plankton, if nutrients concentration is not limiting [113-115].

It is expected that the warming trend will move forward the onset of spring bloom in high latitude and temperate areas [116] and will accelerate the spread and abundance of subtropical species to temperate regions all over the world. *Cylindrospermopsis raciborskii* is probably the best known example. It was originally found in the tropics, in central Africa and Australian lakes; at present, starting from the mid 90s, it has been reported in several temperate areas, in Europe and central America. Climate change is one of the factors considered in explaining its diffusion [117, 118]. Notwithstanding the areal expansion, the risk due to the exposure to *C. raciborskii* cannot be directly inferred and it is not straightforward that it will increase, since the toxins produced in different continents are different: the Australian strains produce cylindrospermopsin [119], whereas the Brazilian or European strains produce saxitoxins and a still unknown toxin, respectively [120, 121].

Another example of expected larger diffusion of tropical species is that of *Trichodesmium* spp. in seawater. Temperature dependence of O_2 flux and respiration seems to be the main reason for this non heterocystous N_2 fixing species to be dominant in subtropical and tropical ocean regions [122]. The reduced O_2 flux in warm seawater and the high respiration rate at high temperature allow *Trichodesmium* to outcompete other heterocystous species. It has been estimated that the poleward shift of the 20 °C isothermal as a consequence of a 3 °C increase by 2090, which poses a lower limit to the distribution of *Trichodesmium*, will expand its range of 11%, even if this will be counteracted by the reduction of 16% of its thermal niche at the tropics, where temperature is expected to exceed 30 °C, corresponding to suboptimal growth rates [123]. It is therefore reasonable to expect a poleward shift of this species, being all the other parameters unchanged.

Complex interactions between direct and indirect effects of temperature are more often the cause of shifts in community composition and changes in dominant species. Increasing thermal stability and stratification in deep, eutrophic lakes will favor the proliferation of buoyant cyanobacteria, like *Microcystis*, *Anabaena*, *Aphanizomenon*, and elevate the chance for the development of surface blooms [124]. Milder winter in temperate eutrophic lakes, may indirectly favor also Oscillatoriales bloom, by inducing a complex shift in phytoplankton community composition [125].

The observed significant increase in cyanobacterial biomass over the years in the Baltic Sea, composed mainly by *Aphanizomenon flos-aquae*, *Nodularia spumigena* and *Anabaena* sp., has been correlated to increased temperature, to variation in the hydrology, and to nutrients inputs [126]. Hence a general higher exposure of aquatic organisms, and possibly of their consumers, human beings included, can be foreseen.

Data available indicate that the prolonged thermal stratification, due to warming climate, would favor also species like *Planktothrix rubescens* [127], which are usually more adapted to colder water.

Data on incresing temperature effects on toxicity are controversial, and depend on the co-occurrence of other parameters, like light and nutrients [128-129]. Davis *et al.* [129] showed that in 4 lakes in Northeast US, higher temperature (and phosphorus concentration) favored an increase of microcystins MCs production by stimulating the growth rate of *Microcystis* spp. toxic cells [129]. During the last decade, the rise of minimal surface temperature of many reservoirs and water basin in South Africa seems to be the direct cause of the proliferation and bloom of toxic cyanobacteria, since there has been a correlated increase of animal mortalities in several areas [130].

In *Oscillatoria agardhii* isolated from Finnish lakes MCs production rate peaked at optimum growth temperature in one strain (25 °C) and was constant in the range 15-25 °C in a second one. However, for both strains, growth and toxin production decreased at 30 °C [131]. On the contrary, for *Microcystis aeruginosa* increasing temperature from 18 °C to 28 °C, yielded higher growth rates, but reduced toxicity of the cultured strains [132]. Also the production of *cylindrospermopsin* CYN seems to be inhibited by higher temperature in tropical *Cylindrospermopsis* [119] and in temperate *Aphanizomenon* [133].

Additionally, temperature can impact on toxicity by affecting the variants profile of toxin production, as it happened in an *Aphanizomenon* sp. cultured strain, which following an increase of T from 22 to 28 °C, switched towards the production of the more toxic variant among the 4 detected toxins [134].

In two strains of *Anabaena* from a lake in Southern Finland, temperature higher and lower than the optimum (25 °C) decreased the total amount of MCs production. In addition the profile of MCs variants changed: high light and high temperature favored the production of the less toxic -RR variant, while an increase in -LR variant was observed at lower temperature [135].

Droughts

The likely longer dryness periods, increasing in some regions at mid-latitudes and in the dry tropics, some of which already water-stressed areas, may influence cyanobacterial proliferation by increasing nutrient availability (higher concentrations due to surface water evaporation in summer) and reducing water bodies flow (thus increasing the areas of still waters in which cyanobacterial growth is easier). One of the largest bloom of *Anabaena circinalis* involved more than 1000 km of one of Australia's major river systems, in a very low flow conditions, concurrent with high nutrients concentration and high temperature [136].

Another important consequence of droughts is that rainfall following period of dryness, can transport cyanotoxins into groundwater, beneath parched and cracked soil, thus contaminating very precious sources of drinking water.

Changes in phytoplankton communities of coastal environment exposed to a reduction in freshwater discharge and to saltwater intrusion, can eliminate more sensitive species and favor more tolerant cyanobacteria and expose aquatic organisms to higher concentration of dissolved cyanotoxins [103].

In Nebraska lakes a lower water quality due to the presence of MCs, associated with the drought conditions and lower nitrogen:phosphorus ratios [137], was reported during 2004, concomitant with dogs, wildlife and livestock deaths, and more than 50 accounts of human skin rashes, lesions, or gastrointestinal illnesses.

CONCLUSIVE REMARKS

Reviewed data show that increase of water temperature, heavy rains, floods and droughts will increase the distribution and patterns of human exposures to pathogens, chemicals and cyanobacteria. Several studies have shown the association between heavy rainfalls/floods and outbreaks of waterborne diseases. The number and severity of these outbreaks are destined to increase. In particular, zoonotic infections will likely increase as a consequence of washing into water of wild animal and livestock faeces due to heavy rain falls and floods. Different strains of enteric viruses and protozoa will increase their concentrations in water bodies representing etiological pathogens not signaled by conventional faecal contamination indicators.

Several outbreaks have been reported due to groundwater contamination by pathogens as a consequence of drought, that has lowered the water table, resulting in changes in underground water flows of surface water into groundwater.

Increasing temperature could favor the growth of some pathogenic indigenous bacteria, like *Vibrio parahaemolyticus*, *V. vulnificus* and *V. alginolyticus*, which are already among the most common etiological agents responsible for diseases transmitted by seafood consumption in US. Increasing temperatures are expected to expand the range and increase the prevalence of *V. cholerae* and cholera, whose diffusion will also be favored by the destructive effects of floods and hurricane on water distribution systems.

Heavy rainfalls and floods will increase the release of chemical constituents from minerals and their concentrations in natural waters, including that of elements like As and F, that at high levels are dangerous for human health. Similarly they will increase concentrations of precursors of DBPs, leading to higher exposures through drinking water consumption. Higher temperature in the oceans may increase human exposure to methyl mercury, especially in geographical areas where the population diet is based on seafood, increasing the number of people above the tolerable weekly intake defined by WHO.

Increased temperatures and floods will promote the formation rate of DBPs.

Heavy rainfalls and floods will expand the distribution of freshwater cyanobacteria into brackish and coastal waters, by physical transport of cyanobacterial blooms into estuaries and by altering the chemical and physical conditions of those areas, ultimately affecting the composition of phytoplankton. These effects will increase the risk of exposure of seafood to cyanotoxins, which is going to be one of the new scenarios of exposure.

Also temperature will affect the geographical distribution of tropical toxic species, that will move towards temperate latitudes, where water temperature is expected to increase. Many deep eutrophic lakes are likely to be dominated by buoyant cyanobacteria, by the protraction of water stratification, which will prolong the period of exposure.

Of course the above predictions do not consider the role of management measures that can be implemented to counteract the worsening scenario, especially to mitigate the effects of extreme events. For these factors, the main focus is identifying regions most at risk of flooding and preparing plans for responding and mitigating the main consequences, as stated in the European directive on management of floods [138].

Many Countries worldwide are strengthening their ability to cope with these events improving their early warning systems, vulnerability assessment and response plan to emergency, still we need a more coordinated approach among different operators (environmental, public and veterinary health, water managers, utilities sectors, land use managers) to counteract water unsafety.

WBDs health surveillance, post extreme events environmental and biota monitoring of affected areas, efficiency of waste water treatment are also crucial areas of action for adaptation measures as well as training of professionals involved at local level on new risk scenarios of WBDs.

If the increase of temperature and extreme events is a result of global warming due to human activities, losses, damages, increased burden of diseases could continue rising indefinitely. Long term measures to counteract the impact of climate change on human health rely on a sound strategy aimed at reducing green house gases emissions.

Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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Section II

Publications from International Organizations on Public Health

Indexes of the Volume

Publications from International Organizations on Public Health

Edited by
Anna Maria Rossi

European Food Safety Authority (EFSA)

Guidance on risk assessment for animal welfare. EFSA Journal 2012;10(1):2513 (30 p.) doi:10.2903/j.efsa.2012.2513 The document provides methodological guidance to assess risks for animal welfare, considering the various husbandry systems, management procedures and the different animal welfare issues. The terminology for the risk assessment of animal welfare is described. Risk assessment should not be carried out unless the relevant welfare problem is clearly specified and formulated. The major components of the problem formulation are the description of the exposure scenario, the target population and the conceptual model linking the relevant factors of animal welfare concern. The formal risk assessment consists of exposure assessment, consequence characterisation, and risk characterisation. The systematic evaluation of the various aspects and components of the assessment procedure aims at ensuring its consistency. All assumptions used in problem formulation and risk assessment need to be clear. This also applies to uncertainty and variability in the various steps of the risk assessment. The choice between qualitative, semi-qualitative or quantitative approaches should be made based on the purpose or the type of questions to be answered, data, and resource availability for a specific risk assessment. Quantitative data should be used whenever possible. Positive effects on welfare (benefit) could be handled within the framework of risk assessment if the analysis considers factors as having both positive and negative effects on animal welfare. The last section details the main components of risk assessment documentation.

"Schmallenberg" virus: analysis of the epidemiological data and assessment of impact. EFSA Journal 2012;10(6):2768 (89 p.) doi:10.2903/j.efsa.2012.2768 This scientific report provides an overall assessment of the impact of the infection on animal health, animal production and animal welfare of the provisionally named "Schmallenberg" virus (SBV) first detected in Germany. In Europe, 3745 holdings have been reported with SBV cases confirmed by laboratory testing across several Member States, mid May 2012. EFSA reviewed the epidemiological reports noting that SBV has been detected in cattle, sheep, goats and a bison. SBV antibodies have been detected in deer and no other species are known to be affected. EFSA also confirms that new studies support the initial assessment undertaken by the European Center for Disease Control and Prevention, that it is very unlikely that SBV poses a risk to humans.
In terms of transmission routes, recent entomological investigations have identified SBV in field samples of biting midges of the *Culicoides obsoletus* group. Currently there is no evidence of any other route of transmission other than transplacental or vector borne routes. EFSA coordinated the collation of SBV epidemiological data during 2011-2012 in order to obtain comparable data for Europe. The maximum proportion of reported sheep holdings with SBV confirmed was 4% per country and 7.6% per region while for cattle less than 1.3% of holdings were reported as SBV confirmed at both country and regional level. In order to assess the impact of SBV(spatial and temporal spread, proportion of affected holding and potential projection of arthrogryposis hydranencephaly syndrome cases) three models were used…
continues at http://www.efsa.europa.eu/en/efsajournal/doc/2768.pdf

Food and Agriculture Organization of the United Nations (FAO)

Investigating the role of bats in emerging zoonoses. Balancing ecology, conservation and public health interest. Rome: Food and Agriculture Organization of the United Nations, 2011, 182 p. ISBN 978 92 510 7028 4 (*FAO Animal Production and Health Manuals*; 12) FAO number: I2407/E
US $ 40.00. This manual on bats and their role in emerging infectious diseases in animals and humans underlines their important role in maintaining the delicate balance in ecosystems that support human, plant and animal life. This document is a manual meant to be used by epidemiologists, wildlife offi-

cials, farmers, livestock veterinarians, zoologists, and any number of different professionals who might be coming into increased contact with bats. It is a hands-on reference to their history, biology, monitoring and handling them, especially amid growing evidence they can be a route for introduction of emerging diseases in livestock and humans.

Quality assurance for animal feed analysis laboratories. Rome: Food and Agriculture Organization of the United Nations, 2011, 192 p. (*FAO Animal Production and Health Manuals*; 14) ISBN 978 92 510 7050 5 FAO Number: I2441/E US $ 40.00. Every sector of the livestock industry, the associated services and the wellbeing of both animals and humans are influenced by animal feeding. This document gives a comprehensive account of good laboratory practices, quality assurance procedures and examples of standard operating procedures as used in individual specialist laboratories. The manual will be useful for laboratory analysts, laboratory managers, research students and teachers and it is hoped that it will enable workers in animal industry, including the aquaculture industry, to appreciate the importance of proven reliable data and the associated quality assurances approaches.

Challenges of animal health information systems and surveillance for animal disease and zoonoses. Rome: Food and Agriculture Organization of the United Nations, 2011, 136 p. (*FAO Animal Production and Health Proceedings*; 14) ISBN 978 92 510 7034 5 FAO Number: I2415/E US $ 38.00. Animal disease surveillance is key to improving disease analysis, early warning and predicting disease emergence and spread. As a preventive measure, disease surveillance is aimed at reducing animal health-related risks and major consequences of disease outbreaks on food production and livelihoods. Early warning systems are dependent on the quality of animal disease information collected at all levels via effective surveillance; therefore, data gathering and sharing is essential to understand the dynamics of animal diseases in diverse agro-ecological settings to support effective decision-making to prevent disease and for emergency response.

For more information visit the FAO publication catalogue http://www.fao.org/icatalog/inter-e.htm

Organisation for Economic Co-operation and Development (OECD)

Mental health and work. Sick on the job? Myths and realities about mental health and work. Paris: OECD Publishing 21 Feb 2012, 212 p. ISBN 978 92 641 2451 6 OECD Code: 812011181P1 € 50.00/US $ 70.00 . The costs of mental ill-health for the individuals concerned, employers and society at large are enormous. Mental illness is responsible for a very significant loss of potential labour supply, high rates of unemployment, and a high incidence of sickness absence and reduced productivity at work. In particular, mental illness causes too many young people to leave the labour market, or never really enter it, through early moves onto disability benefit. Today, between one-third and one-half of all new disability benefit claims are for reasons of mental ill-health, and among young adults that proportion goes up to over 70%. Indeed, mental ill-health is becoming a key issue for the well-functioning of OECD's labour markets and social policies and requires a stronger focus on policies addressing mental health and work issues. Despite the very high costs to the individuals and the economy, there is only little awareness about the connection between mental health and work, and the drivers behind the labour market outcomes and the level of inactivity of people with mental ill-health. Understanding these drivers is critical for the development of more effective policies. This report aims to identify the knowledge gaps and begin to narrow them by reviewing evidence on the main challenges and barriers to better integrating people with mental illness in the world of work.

Livestock diseases. Prevention, control and compensation schemes. Paris: OECD Publishing 30 Aug 2012, 204 p. ISBN 978 92 641 7875 5 OECD Code: 512012081P1 € 60.00/US $ 84.00. This report is an overview of the management of risk due to livestock diseases, a potentially catastrophic type of risk that can have strong external effects given its links to the food chain and to human health. Animal disease, primarily in farmed livestock, has long been a policy concern for food safety reasons and the high economic losses it can engender. The globalisation of trade and human movement, and sensitivities to food safety, enhance the relevance and complexity of disease control for terrestrial livestock. Outbreaks – or even rumours of an outbreak – can result in widespread consumer alarm, disruption of trade, and severe effects on incomes, not to mention the human cost of illnesses and deaths arising from animal disease.

Health at glance: Europe 2012. Paris: OECD Publishing 19 Nov 2012, 160 p. ISBN 978 92 641 8360 5 OECD Code: 812012121P1 € 30.00/US $ 42.00. This second edition of *Health at a Glance: Europe* presents a set of key indicators of health status, determinants of health, health care resources and activities, quality of care, health expenditure and financing in 35 European countries, including the 27 European Union member states, 5 candi-

date countries and 3 EFTA countries. The selection of indicators is based largely on the European Community Health Indicators (ECHI) shortlist, a set of indicators that has been developed to guide the reporting of health statistics in the European Union. It is complemented by additional indicators on health expenditure and quality of care, building on the OECD expertise in these areas. Each indicator is presented in a user-friendly format, consisting of charts illustrating variations across countries and over time, a brief descriptive analysis highlighting the major findings conveyed by the data, and a methodological box on the definition of the indicator and any limitations in data comparability.

World Health Organization (WHO)

Global tuberculosis report 2012. Geneva: World Health Organization. 2012, 280 p. ISBN 978 92 415 6450 2 Order number: 11507659Sw.fr.40.00/ US $ 48.00. This is the seventeenth global report on tuberculosis (TB) published by WHO in a series that started in 1997. It provides a comprehensive and up-to-date assessment of the TB epidemic and progress in implementing and financing TB prevention, care and control at global, regional and country levels using data reported by 198 countries that account for over 99% of the world s TB cases.The introductory chapter provides general background on TB as well as an explanation of global targets for TB control, the WHO s Stop TB Strategy and the Stop TB Partnership s Global Plan to Stop TB 2011 2015. The remaining six chapters of the report cover the burden of disease caused by TB; case notifications and treatment outcomes; financing TB care and control; diagnostics and laboratory strengthening for TB; addressing the co-epidemics of TB and HIV; and research and development for new TB diagnostics, drugs and vaccines. The four annexes of the report include a thorough explanation of methods used to estimate the burden of disease caused by TB, one-page profiles for high TB-burden countries, and tables of data on key indicators for all countries.

Dufour A, Bartram J, Bos R, *et al.* **Animal waste, water quality and human health.** Geneva: World Health Organization. 2012, 486 p. *(WHO Emerging Issues in Water & Infectious Disease Series)* ISBN 9789241564519Order number: 11500849Sw.fr.90.00/ US $ 108.00. Domestic animals contaminate recreational waters and drinking-water sources with excreta and pathogens; but this threat to public health is inadequately understood and is insufficiently addressed in regulations. More than 85% of the world's faecal wastes is from domestic animals such as poultry, cattle, sheep and pigs. These animals harbor zoonotic pathogens that are transported in the environment by water, especially runoff. However little information exists on health effects associated with exposure to this potential hazard to human health; and water standards focused on control of human faecal contamination do reflect the contribution of non-human faecal contamination to risk. Does compliance with current monitoring practices using microbial indicators provide protection against animal and bird sources of fecal contamination?
Prepared with contributions from a group of international experts, this book considers microbial contamination from domestic animal and bird sources and explores the health hazards associated with this microbial contamination and approaches to protecting public health. This book will be of interest to regulators with responsibility for recreational waters, drinking-water quality and water reuse; policymakers working in water quality, public health and agriculture; decision makers responsible for livestock management; and scientists and practitioners concerned with many affected subjects.

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Section I

Instructions to authors

Annali dell'Istituto Superiore di Sanità is a peer reviewed science journal which publishes research articles in biomedicine, translational research and in many other disciplines of the health sciences. The journal includes the following material: original articles, reviews, monographic issues, commentaries, brief and technical notes, book reviews. *Annali* follows the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, issued by the International Committee of Medical Journal Editors (ICMJE).

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- cover letter where the authors declare that the manuscript has not been published or submitted for publication elsewhere;
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- tables and figures;
- author's contribution statement (individual contribution to the manuscript);
- conflict of interest statement (a conflict of interest exists when authors or their institutions have financial or personal relationship with other people or organizations that could inappropriately bias conduct and findings of the study).
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Receipt of author's paper will be acknowledged by an e-mail containing an identification number which should be used in future correspondence.

Review procedure

Each paper submitted to *Annali* is subjected to the following procedures:

- it is reviewed by the Editor-in-Chief for general suitability;
- if it is judged suitable, qualified referees are selected and a peer review process takes place (occasional contributions, such as commentaries, papers submitted on invitation, etc. are accepted without peer review);
- based on the recommendations of the referees and replies of the authors, the Editor-in-Chief decides whether the article should be accepted, modified or rejected;
- once the paper has been accepted, authors will receive proofs from the editorial office, which should be corrected and returned (usually within three working days).

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Please ensure that your manuscript follows these guidelines. For presentation of monographic issues refer to the instructions available online at www.iss.it/anna.

Manuscripts should be written as concisely as possible to allow a clear understanding of the text.

The title, on a separate sheet, should be followed by the complete name of the authors, their affiliations – in the original language – town and country. The name and address, telephone and e-mail of the corresponding author should also be indicated. On the same page a running head of no more than 40 characters (including spaces) should be included.
Original articles should normally be organized into different sections (*i.e.*: Introduction, Materials and methods, Results, Discussion, Conclusions).

Each article should be accompanied by:

- a structured abstract both in English and in Italian (with Italian translation of the title) of about 120 words, presented on separate sheet;
- key words in English and Italian, up to a maximum number of five (MeSH headings, whenever possible. Refer to: www.nlm.nih.gov/mesh/meshhome.html and to www.iss.it/site/mesh for the Mesh Italian translation).

Tables and figures should be kept to a minimum and be presented only if necessary.

Authors should deal responsibly and effectively with security issues that might be raised by their papers (see: Statement on Scientific Publication and Security *Science* 2003;299:1149).

Authors are also encouraged to include data disaggregated by sex (and, whenever possible, by race) and provide a comprehensive analysis of gender and racial differences. The authors should include the number and percentage of men, women and, if appropriate, transgender persons who participated in the research study. Anatomical and physiological differences between men and women (height, weight, body fat-to-muscle ratios, cell counts, hormonal cycles, etc.), as well as social and cultural variables (socio-economic, education, access to care, etc.), should be taken into consideration in the presentation of data and/or analysis of the results. Authors should consider gender issues also in the authorship.

The name of the bioresource (and identifier, if available) who provided samples/data useful for the conduct of the study should be reported in extense, either in the *Material and Methods* section or in the *Acknowledgements.*

Length of the text

To provide a text that meets the requirements of our publication:

- the *editorial* should be no longer than 1000 words;
- the *commentary,* 2000 words;
- the *brief note*, 3000 words, including about 15 references, one table and one figure;
- the *article,* 6000 words, including about 40 references, three tables and two figures;
- the *review* should be no longer than 10 000 words, including about 100 references, four tables and three figures.

Formatting guidelines

Text

- Use Times New Roman font, 10 point, single spaced;
- do not use the automated features of your application (endnotes, headers, footers, especially for references);
- type a single space at the end of each sentence;
- avoid using bold characters to emphasise words or sentences within the text;

Indicate clearly titles of chapters and subchapters avoiding numbering.

Tables and figures

They should be understandable also without reference to the text and should be numbered in Arabic numerals in a consecutive and independent way according to their citation within the paper.

Tables should be presented on a separate sheet and preceeded by a title. Each column within the table should have a heading. Abbreviations should be reported in full in the legend.

Figures should be loaded as separate files. The following file formats are acceptable: JPEG, TIFF or EPS.

Vectorial images (flow charts, schemes, and other non bitmap material) should be in Macromedia Freehand, Adobe Illustrator, Microsoft Power Point.

Figures are redrawn into the *Annali* style by our in-house illustrators.

Photographs must have a minimum resolution of 300 dpi, width 107 mm and presented in black and white (an extra-cost is due by the author for each colour photograph).

Captions should be presented on a separate sheet and contain a sufficient explanation of their object.

Conventions

All Latin or foreign words should be in italics. The authors should use internationally accepted abbreviations. All abbreviations should be spelled out in full the first time they occur in the text, followed by the shortened term in parentheses; afterwards use the abbreviation only.

References

All references in the text must be numbered in square brackets, *i.e.* [1, 2, 3-6], and mentioned at the end of the article in the order in which they are quoted. They should conform to the "Uniform Requirements for Manuscript Submitted to Biomedical Journals" (known as *Vancouver style,* www.icmje.org), with some variations according to the following examples.

Titles of periodicals should be abbreviated in accordance with the Medline abbreviation of the US National Library of Medicine (www.nlm.nih.gov/bsd/aim.html). Online journal articles can be cited using, in addition to the complete citation, the DOI number.

Articles in journal

Bozzuto Giuseppina, Ruggieri P, Molinari A. Molecular aspects of tumor cell migration and invasion. *Ann Ist Super Sanità* 2010;46(1):66-80.

Books and chapters in a book

Godlee F, Jefferson T. *Peer review in health sciences*. London: BMJ Books; 1999.

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