Environmental Science
Processes & Impacts

CRITICAL REVIEW

View Article Online
View Journal | View Issue

Cite this: *Environ. Sci.: Processes Impacts*, 2014, **16**, 2145

The interaction of human microbial pathogens, particulate material and nutrients in estuarine environments and their impacts on recreational and shellfish waters

Shelagh K. Malham,*[a] Paulina Rajko-Nenow,[a] Eleanor Howlett,[a] Karen E. Tuson,[a] Tracy L. Perkins,[b] Denise W. Pallett,[c] Hui Wang,[c] Colin F. Jago,[d] Davey L. Jones[e] and James E. McDonald[b]

Received 10th January 2014
Accepted 7th July 2014

DOI: 10.1039/c4em00031e

rsc.li/process-impacts

Anthropogenic activities have increased the load of faecal bacteria, pathogenic viruses and nutrients in rivers, estuaries and coastal areas through point and diffuse sources such as sewage discharges and agricultural runoff. These areas are used by humans for both commercial and recreational activities and are therefore protected by a range of European Directives. If water quality declines in these zones, significant economic losses can occur. Identifying the sources of pollution, however, is notoriously difficult due to the ephemeral nature of discharges, their diffuse source, and uncertainties associated with transport and transformation of the pollutants through the freshwater–marine interface. Further, significant interaction between nutrients, microorganisms and particulates can occur in the water column making prediction of the fate and potential infectivity of human pathogenic organisms difficult to ascertain. This interaction is most prevalent in estuarine environments due to the formation of flocs (suspended sediment) at the marine-freshwater interface. A range of physical, chemical and biological processes can induce the co-flocculation of microorganisms, organic matter and mineral particles resulting in pathogenic organisms becoming potentially protected from a range of biotic (*e.g.* predation) and abiotic stresses (*e.g.* UV, salinity). These flocs contain and retain macro- and micro- nutrients allowing the potential survival, growth and transfer of pathogenic organisms to commercially sensitive areas (*e.g.* beaches, shellfish harvesting waters). The flocs can either be transported directly to the coastal environment or can become deposited in the estuary forming cohesive sediments where pathogens can survive for long periods. Especially in response to storms, these sediments can be subsequently remobilised releasing pulses of potential pathogenic organisms back into the water column leading to contamination of marine waters long after the initial contamination event occurred. Further work, however, is still required to understand and predict the potential human infectivity of pathogenic organisms alongside the better design of early warning systems and surveillance measures for risk assessment purposes.

Environmental impact

Microbial pathogens enter estuaries *via* several point and diffuse sources that include agricultural runoff, wildlife excrement, septic tank and sewage discharges. Human pathogens subsequently accumulate within sediments and on suspended sediments (flocs) in riverine and estuarine waters, where deposition of flocculated particles promotes reservoirs of potentially pathogenic bacteria and viruses in the sediment. Macronutrient fluxes play a critical role in floc formation, sediment dynamics and pathogen survival, as it is well established that nutrient availability and sediment association enhance the survival and persistence of pathogens. Pathogen transport in flocs affects water quality and shellfish hygiene, which may impart significant impacts upon commercial and recreational activities in estuarine and coastal environments with potential implications for human health.

[a]*Centre for Applied Marine Science, Bangor University, Menai Bridge, Anglesey, LL59 5AB, UK*
[b]*School of Biological Sciences, Bangor University, Deiniol Road, Bangor, LL57 2UW, UK*
[c]*Centre for Ecology and Hydrology, Benson Lane, Wallingford, Oxfordshire, OX10 8BB, UK*
[d]*School of Ocean Sciences, Bangor University, Menai Bridge, Anglesey, LL59 5AB, UK*
[e]*School of Environment, Natural Resources and Geography, Bangor University, Deiniol Road, Bangor, LL57 2UW, UK*

1. Introduction

Estuaries are highly productive and dynamic areas at the transition between river and marine environments. Consequently, estuarine and coastal areas receive and are involved in processing a large proportion of the water moving through a catchment. Crucially, estuaries and coasts are areas of vital

aquatic resources, providing food and habitat for fish and shellfish, food for human consumption, areas for tourism and recreation, in addition to a host of other ecosystem services.[1,2] The point and diffuse inputs of pollutants to the estuary and coastal zone include urban and agricultural runoff, sewage and contaminants, all of which have significant impacts on these ecologically and economically important areas.[3,4]

Of particular importance to human health within estuaries and coasts are the presence and survival of human pathogenic microorganisms (bacteria, protozoa and viruses). These pathogens are often associated with sediments and flocs (suspended sediments) and typically arise from contamination of fresh and marine waters with human sewage. Within Europe, bathing beaches and shellfish waters are monitored for compliance with European Union (EU) standards (*e.g.* Bathing Water Directive (76/160/EEC, revised by Directive 2006/7/EC) and EC Shellfish Waters Directive 2006/113/EC) although the latter of these directives was repealed in 2013 by the EU Water Framework Directive (WFD) (2000/60/EC). The EU WFD will provide similar levels of protection as given by the repealed directive. For bathing water, higher microbial standards (*Escherichia coli* and intestinal enterococci) for water quality in particular, will be required.

2. Microbial pathogens associated with recreational and shellfish-growing waters

A wide range of bacterial, protozoan and viral pathogens have been responsible for waterborne[5] and shellfish-borne illness (Table 1).[6] Human pathogens present in estuarine environments are mainly derived from human or animal faeces. These pathogenic microorganisms can be transported from upstream sources to estuarine and coastal waters, especially during heavy rain or flood events that may then impact on recreational and shellfish growing waters. Water-related activities such as swimming, boating or other water sports in faecally-contaminated waters can pose a risk to human health.[7] However, bather shedding can also be a source of potentially pathogenic microorganisms in coastal waters. Bivalve molluscan shellfish are filter-feeding organisms that can accumulate pathogens from faecally contaminated estuarine waters and may present a health risk when consumed raw or only lightly cooked.[8]

2.1 Bacterial pathogens

Salmonella spp. are naturally occurring bacteria found in the intestinal tract of humans, animals, birds and reptiles.[9] Several subspecies of *Salmonella enterica* exist and have been implicated in small-scale foodborne outbreaks. However, unlike most other *Salmonella* spp., *Salmonella typhi* and *Salmonella paratyphi* have been responsible for larger outbreaks of waterborne illness.[10] The majority of reported salmonellosis is caused by *S. enterica* that is generally associated with food products. Typhoid fever was frequently associated with the consumption of sewage-contaminated oysters at the beginning of the 20th century, with several large outbreaks reported by the USA associated with 150 deaths.[6]

Verocytotoxigenic (VTEC) *E. coli* O157 produces potent bacteriophage-encoded verocytotoxin or Shiga-like toxins and is often responsible for severe acute haemorrhagic diarrhoea that can lead to death.[11] *E. coli* O157 is common in the intestine of healthy cattle and ruminants, but is associated with severe outbreaks of human enteric illness. Stream water, beaches and vegetable fields can become contaminated with cattle faeces containing *E. coli* O157, which becomes mobilised in run-off from cattle pasture. *E. coli* O157 infections were previously associated with the consumption of vegetables[12] contaminated by animal feaces.[13] Waterborne transmission of *E. coli* O157 has been associated with swimming in recreational lakes,[14] drinking water from private wells[15] and municipal water supplies.[16]

Shigella species are Gram-negative bacteria found in the gastrointestinal tract of humans. *S. sonnei* is the most common species of *Shigella* implicated in gastrointestinal illness in England and Wales, and is mainly associated with foreign travel.[17] Inadequate disposal of human sewage into recreational waters and/or lack of effectively treated water supply were previously associated with outbreaks of *Shigella* in the USA.[5] An oyster-related outbreak of *S. sonnei* infection among 24 individuals occurred in Texas and was associated with the disposal of faecal waste overboard from the oyster harvesting boat.[18]

During the period 1992–2003, *Campylobacter* spp. were responsible for 14% of all waterborne outbreaks of 89 reported in England and Wales, and were mainly associated with private water supplies.[15] However, *Campylobacter jejuni* has been frequently detected in wastewaters and was isolated from environmental water.[19,20]

Although many pathogenic bacteria are found in human sewage and animal faeces, bacterial illness associated with shellfish consumption appears to constitute a minimal public health hazard in Europe.[8] The implementation of various national monitoring programmes in developed countries has significantly reduced incidences of bacterial illness. Where such monitoring programme exists shellfish-mediated bacterial illness is generally associated with illegally harvested shellfish from contaminated areas or inadequate post-harvest treatment processes.

Unlike the previously mentioned allochthonous bacterial pathogens, *Vibrio* spp. are native to both marine and estuarine environments and their occurrence in seawater is typically unrelated to faecal pollution. *Vibrio* spp. are halophilic, non-spore forming bacteria that grow in warm water (>18 °C) of low or moderate salinity.[21] They are fast growing bacteria in nature and can multiply readily in oysters post-harvest when not properly refrigerated. *Vibrio* spp. can cause infection through exposure to seafood and seawater and produce a wide range of clinical symptoms. *Vibrio vulnificus* can result in septicaemia with a high mortality rate, others species are associated with gastroenteritis of varying severity (Table 1).[6]

Vibrio vulnificus and *V. parahaemolyticus* have recently been detected in oysters at retail during market survey in the USA; oysters sampled from the Gulf Coast had the highest numbers

Table 1 Microbial pathogens associated with recreational water or/and shellfish-related illness

Pathogen	Incubation	Symptoms/illness	Source of water contamination
Bacteria			
Campylobacter spp	2–4 days	Cramps, abdominal pain, diarrhoea (with or without blood or fecal leukocytes), chills, and fever, sometimes leading to Guillan-Barré syndrome	Animal/bird faeces/slurry
E. coli O157	1–8 days (average: 3–4 days)	Abdominal cramps and tenderness and bloody diarrhoea, sometimes leading haemolytic–uremic syndrome, renal failure and death	Animal faeces/slurry
Salmonella typhi and *Paratyphi*	1 to 14 days (average 3 to 5 days)	Typhoid or paratyphoid fever: headache, central nervous signs, malaise, anorexia, splenomegaly, and rose spots on the trunk, sometimes cause septicaemia	Human faeces/sewage
Salmonella enterica (various dirserogroups)	6–72 hours	Diarrhoea, fever, and abdominal cramps	Human faeces/sewage or animal bird faeces/slurry
Vibrio vulnificus	16 hours	Septicaemia: malaise, chills, fever, prostration, cutaneous lesions, fatalities occur	Estuaries and marine waters
Vibrio parahaemolyticus	2–48 hours	Abdominal pain, diarrhoea, nausea, vomiting, chills, headache	Estuaries and marine waters
Vibrio cholerae O1 and O139 serotypes	1–5 days	Profuse, watery diarrhoea (rice–water stools), vomiting, abdominal pain, dehydration	Human faeces/sewage
Shigella spp	1–3 days	Abdominal pain, diarrhoea, bloody & mucoid stools, fever	Human faeces/sewage
Protozoan			
Cryptosporidium parvum	7–10 days	Profuse and watery diarrhoea, weight loss, nausea; low-grade fever	Human faeces/sewage or animal faeces/slurry
Giardia duodenalis	5–25 days	Diarrhoea, malabsorption and weight loss	Human faeces/sewage
Virus			
Adenoviruses	~10 days	Fever, upper and lower respiratory track symptoms, conjunctivitis, gastroenteritis, eye infection	Human sewage
Norovirus	24–48 hours	Diarrhoea, nausea, vomiting, abdominal pain, abdominalcramps	Human sewage
Hepatitis A	15–50 days	Fever, malaise, lassitude, anorexia, nausea, abdominal pain, jaundice,	Human sewage
Echoviruses	2–4 days	Gastroenteritis, encephalitis, meningitis	Human sewage
Coxackieviruses	2–12 days	Meningitis, pharyngitis, conjunctivitis, encephalitis	Human sewage

of both *Vibrio* species compared to other regions in USA during 2007.[22] Recreational water users with open wounds have been infected previously by *Vibrio vulnificus* in the Gulf Coast states, USA[23] and in Denmark during remarkably warm summer.[24]

2.2 Protozoan pathogens

The transmission of protozoan parasites *via* seafood is very rarely reported; however, protozoan-contaminated water is a frequent cause of outbreaks.[25] Waterborne outbreaks of *Cryptosporidium parvum* and *Giardia duodenalis* are well documented and have been associated with drinking water,[15] swimming pools, water parks, lakes, rivers and streams.[23] *Cryptosporidium* oocysts are excreted by infected humans or animals and can survive outside the body for long periods of time. *Giardia* cysts are widespread in the environment; being isolated from surface water, coastal beaches, rivers used for recreational activities and swimming pools.[10] The protozoan parasites such as *Cryptosporidium* spp. and *G. duodenalis* have the potential to be accumulated by shellfish from the surrounding waters and retain their infectivity for prolonged periods in shellfish.[26] However, to date, one oyster-associated outbreak of *giardiasis* has been reported.[27]

2.3 Viruses

Enteric viruses are typically released in large number (up to 10^{11} per gram) in the stool of infected humans; therefore, direct or indirect faecal contamination of surface, ground and marine waters are the main source of human viruses in the environment. In rural settings, run-off from agricultural practices may account for a significant portion of viruses detected in groundwater.[28] In urban settings, intense periods of rainfall can overwhelm wastewater treatment plants that may result in the discharge of partially or untreated wastewater directly into receiving waters.[29,30] While many different enteric viruses are present in wastewater,[31] epidemiological studies have shown that few have been implicated in waterborne and shellfish vectored illness. Norovirus is a leading cause of viral recreational water–borne outbreaks documented in the literature (45%), followed by adenoviruses (24%), echovirus (18%), hepatitis A virus (7%) and coxsackieviruses (5%).[32] Norovirus is

also the leading cause of shellfish-borne outbreaks, being responsible for 83.7% of outbreaks reported, followed by hepatitis A virus (12.8%).[33] Multiple human enteric viruses such as aichi virus, norovirus, astrovirus, enterovirus and rotavirus were detected in both faecal and oyster samples following an outbreak of gastroenteritis when 205 cases were linked to the consumption of contaminated oyster.[34] However, shellfish-associated outbreaks attributed to rotaviruses, astroviruses, enteroviruses, and adenoviruses have been rarely reported worldwide.[33]

2.4 Unknown and emerging pathogens

Emerging pathogens[35] are a global challenge in the 21st century.[36] Bacteria and rickettsiae are responsible for more than 50% of emerging infectious disease (EID) incidents while viral pathogens are the next most common.[36] Pathogen emergence is affected by environmental factors[37,38] and these factors also contribute to the complexity of EID dynamics[35] in both terrestrial and aquatic environments.[39] Recently advanced genomics techniques[40,41] have led to significant improvements in the level of knowledge about virus communities in the marine environment,[42] and these advances in technology now offer powerful molecular tools[43] that make environmental monitoring of human pathogens possible.[44] Active and effective molecular monitoring not only serves as the foundation of human pathogen surveillance, but also enables the identification and traceability of pollution sources,[45] thus allowing for disease prevention and management.

3. Sedimentary processes and flocculation

3.1 Sediments

Sediments in natural ecosystems are derived from weathering and erosion of rocks, soils and riverbanks as well containing organic material from plant and animal sources.[46] Sediment particle sizes range from very small colloidal particles <1 μm, to large rocks and boulders. Estuarine environments frequently trap large quantities of fine sediment (*i.e.* clay and silt[47]). The amount, type and size distribution of sediment particles can have significant consequences for the sorption, accumulation and transport of pollutants.[48] In comparison to sand particles, silt and clay represent a major sink for pollutants, not only due to their greater capacity to absorb chemicals from the water column[48] but also due to their ability to sorb and protect microbial pathogens.[49]

3.2 Flocculation

Flocs are aggregates of primarily organic detritus, including extracellular polymeric substances (EPS) exuded from aquatic organisms,[50] inorganic particles such as clay and silt, and water that occupies or moves through large pores that develop within the floc.[51] Due to their high, largely negative, surface charge density they have the potential to bind pathogens. Consequently, flocs represent the main vehicle for the transport of organic material from the water column to the sediment. In addition, as they represent a significant reservoir of freshwater, estuarine and marine carbon, they become heavily colonised by hydrolytic aquatic bacteria.[52,53] Extensive hydrolysis of flocs results in the conversion of particulate organic matter (POM) to dissolved organic matter (DOM), altering the sinking properties of aggregates, and allowing DOM to remain in the upper water column where it may also be mineralised.[53] Flocs therefore represent an important driver of food webs and carbon cycling in marine environments.[53,54] Furthermore, it has recently become apparent that flocs also act as a major reservoir for the persistence of human pathogens in aquatic systems.[55–57] The formation of flocs (flocculation) depends on external physical, chemical and biological factors. Flocs account for approximately 80% of the total volume of sediments in suspension within estuaries.[56] They are particularly important because they sink at a faster rate than the individual particles, as particle size is proportional to its settling velocity, and thus increases the flux of matter to the estuary or river bed.

Flocculation is the result of three principal processes: Brownian motion, differential settling and fluid shear[58,59] and is heavily influenced by salinity, sediment concentration and turbulent intensity. Flocs are multidimensional ephemeral fragile structures which form the most significant component of suspended matter in terms of its biogeochemical role.[60] As such, the composition of flocculated material will reflect catchment type and potentially contain complex mixtures of nutrients, macronutrients, contaminants and biological material depending on point and diffuse sources affecting the aquatic environment. In freshwater, flocs consist mainly of clay, silt and organic matter, bonded by fibrilar extracellular polymeric substances (EPS) and other biopolymers secreted by microbes.[61] In estuarine and saline waters, however, flocs are significantly influenced by environmental factors such as salinity, pH, electro-static charge and organic matter content and undergo rapid transformations either flocculating or settling out.[61] The organic matter within an estuary will vary seasonally and between and within catchments.[61] The physical properties of the floc will influence the floc characteristics, which include particle size, porosity and settling rate.[62] Overall, aggregation and sedimentation of flocs are functions of the hydrodynamics and biological environment of the aquatic system[63] as well as particle composition and modes of bonding of the floc.[64] Low to moderate turbulence and high sediment concentrations in the water increases particle encounter and favours flocculation which occurs at slack water.[64] Larger and more porous flocs, have a fast settling rate leading to depositional flux of silt and clays (mud) to the seabed. These deposited flocs provide important intertidal environments within the estuary.[64] The maximum size of a floc is thought to be determined by the scale of turbulent eddies.[65]

3.3 Sedimentary processes in the estuarine environment

The fate of flocs is dependent not only on their composition, but on the physical environment they exist within. Sedimentary processes in the estuary are controlled by its hydrodynamics, which are complicated due to the tides, interface between fresh and saline waters and bathymetry. The field of estuarine physics is far too wide to review here, for a more comprehensive review the circulation of mixed and partially-mixed estuaries is given in

MacCready and Geyer.[66] In short, the currents in estuaries are a result of both the river flow and the incoming/outgoing tide which causes cyclical production of bed-stress and turbulence. Additionally, salt water penetrates into the estuary with the incoming tide, to meet the fresh river water, the interface of which is typically called the Estuarine Turbidity Maximum (ETM), a trap for suspended particles which aids deposition and the formation of flocs. The bathymetry of the basin and the influence of the freshwater–salt water interface typically results in asymmetrical currents which, in flood (incoming) tide dominant systems, produces stronger near-bed stress and turbulence on the flood tide; it should also be noted here that velocities, and thus turbulence, drop to near-zero on the changing tide with important implications for sediment/floc deposition. As a result of the dynamics of the saline inflow, a net inflow of salt can be found under low- to average-river conditions such that the ETM moves gradually further up the estuary; this may only be flushed out with higher than normal river conditions.[67]

The resuspension, deposition and transport of particles in the estuary are controlled by the pattern of bed stress during the tidal incursions, turbulent kinetic energy availability and turbulent length scales.[68] Friction at the river/estuary bed erodes sediments according to a critical bed stress which is determined per grain size (or typically, from a friction coefficient determined by bed type or grain size) resulting in cyclical (semi-diurnal, fortnightly) resuspension events. Cohesive sediments and flocs containing organic matter, however, do not follow this rule as their biochemical bonds require larger stresses to free them. Additionally, the flood tide is able to prevent settling of particles where the ebb tide may not due to the greater amount of turbulent energy within the water column. Due to the lower friction and turbulence on the ebb tide (than the flood), sediments are able to remain flocculated, to a certain degree, and to start to settle, additionally, they may also not be resuspended as they were on the flood. The vertical distribution of particles within the water column, as a result of these tidal asymmetries, can cause sediment pumping[69] or a net retention of particles within the estuary.

Particulate material retained within the estuary is deposited on the bed. Deposition is a function of several factors[68] including: the magnitude of turbulent energy in the water column which maintains particles of a specific size in suspension; advective settlement defined by the settling velocity of the particle (a function of its density and size); turbulent excursions which can deliver sediment to the bed. Conditions at the bed determine the capture of the particles. In the final part of the cycle, the fate of matter in the bed is controlled by its erodibility which is determined by the bonds between the particles there.

4. Nutrient dynamics and impacts in estuarine environments

Estuaries are at the transition zone between the terrestrial and marine environments and provide significant ecosystem services and benefits in terms of nutrient cycling and attenuation.[70] They play a key role in the processing and cycling of nutrients which depends on several interlinked processes not least of which involves the various particulate or dissolved organic and inorganic forms of various nutrients.[71] Over time, however, fluxes of nutrients such as the macronutrients carbon (C), nitrogen (N) and phosphorus (P) have generally increased in European rivers, estuaries and coasts producing negative impacts such as increased algal blooms, eutrophication, poor water quality and organic pollutant loading (*e.g.* endocrine disrupting chemicals) which can threaten ecosystem health.[71] Diffuse and point source nutrient inputs result from human activity such as sewage discharge outfalls, agriculture runoff, septic tanks and industrial effluents.[70] These discharges are often ephemeral and their chemical composition and concentration can vary dramatically over time in response to a multitude of factors (*e.g.* prevailing climate, land use practices, discharge rate *etc.*). This makes it difficult to not only identify the source of nutrient pollution but also to predict the short and long term impact of nutrients on estuarine ecosystems and to devise potential mitigation strategies. Whilst our knowledge of the behaviour and fate of inorganic N and P in estuarine environments are partly understood, our knowledge of organic forms of N and P and dissolved organic C (DOC) remain poor. To a large extent this is due to myriad of chemical compounds that are present in the water column and the difficulty in both their identification and quantification.[72,73] In addition, negative ecological impacts of these compounds can occur at very low concentrations (1–100 nM).[74,75]

The behaviour and fate of nutrient in estuaries is strongly affected by changes in climate (*e.g.* alterations in temperature, wind and hydrological cycle).[76] The fluxes and impacts of these interlinked nutrients through the river, estuary, and coast continuum are difficult to quantify due to the complex nature of estuarine systems (*i.e.* hydrodynamics of the estuary, tidal flushing times and changes in physio-chemical conditions as fresh and saline waters mix).[71,76] Importantly, changes in nutrient reactivity, speciation and particulate and dissolved forms occur as salt and fresh waters mix due to alterations in pH, ionic strength and dissolved organic matter.[76] Flocs and suspended sediments in estuaries form important foci by electrostatically attracting and concentrating dissolved nutrients from the water column. Sedimentation of these particles therefore increases the residence time of nutrients in estuaries.

5. Interactions of microbial pathogens with sediment, suspended particulate material (flocs) and macronutrients

Human microbial pathogens enter the estuarine environment *via* several point and diffuse sources that include surface runoff, wildlife excrement, septic tank outputs and storm and sanitary sewer overflows. However, numerous biological, chemical and physical factors dictate the fate of pathogens in estuarine ecosystems[77] and it is well established that different pathogen types and even strains of the same pathogen possess variable abilities to survive and persist.[78,79] It has been suggested that faecal bacteria (FB) and their associated pathogens do not

survive for long in aquatic ecosystems,[80,81] but many of these studies have focused only on pathogen survival and persistence within the water column. Comparative studies have demonstrated that pathogen densities are several orders of magnitude higher in sediment when compared with the water column,[56,82,83] and survival of FB in sediments is greater when compared to the water column.[84–86] These data support the well-established paradigm that association with particles enhances pathogen survival and distribution in the environment, yet our understanding of the biotic and abiotic processes that mediate pathogen survival and transport in estuarine systems is limited.

5.1. Pathogen–sediment interactions

Association with sediment particles can enhance the preservation of microorganisms by providing them with survival advantages that include shelter from UV radiation[87] and protection from predation.[88] Upon particle adsorption and sedimentation, the survival and persistence of FB is regulated by various factors including organic content of the particles,[89] available nutrients[90] heavy metal content,[91] predation rate from protozoa,[92] competition from other microorganisms present,[93] temperature,[94] salinity,[95] sunlight intensity[80] and seasonal variation.[96] Sediment characteristics can similarly impact FB abundance. For example, Garzio-Hadzick *et al.*[97] and Craig *et al.*[89] revealed slower inactivation rates of FB in sediments that consist of finer particles and comprise a higher organic content. Howell *et al.*[49] found that as sediment particle size and temperature decreased, FB mortality rates declined. It has also been proposed that particular clay types such as montmorillonite provide *E. coli* and possibly other faecal bacteria protection against bacteriophage attack in saline conditions.[98] Amongst others, Davies *et al.*[82] demonstrated that faecal coliforms and faecal streptococci can survive in sediments for long periods of time, suggesting that estuaries represent long term reservoirs for microbial pollution. Furthermore, when the survival rates of faecal coliforms and faecal streptococci have been compared in the presence and absence of protozoan predators (cyclohexamide was added to some microcosms to inhibit protozoan predation of bacteria), both coliforms and streptococci were found to increase in number, suggesting that faecal bacteria are capable of growth in sediments in the absence of predation.[82] Several other studies have also reported the growth of *E. coli*[79,84,90,99,100] and *E. coli* O157 (ref. 79) in sediments. Historically, sunlight was thought to be the single most important factor mediating faecal bacterial die-off in aquatic environments, but recent studies suggest that protozoan predation is the primary driver of FB decay.[79] However, there is contrasting data on the effect of protozoan predation on the survival of *E. coli* O157:H7 strains in the estuarine environment,[79] demonstrating that the intricate relationship between pathogen growth and predation in aquatic environments requires further study.

Sediments also represent a reservoir for the cysts/oocysts of pathogenic protozoa such as *Giardia duodenalis*, *Cryptosporidium* spp. and *Toxoplama gondii.* The interaction of protozoan cysts/oocysts with both organic and inorganic particles promotes their survival, transport and persistence in the environment, in some cases for several months.[101] Medema *et al.*[102] demonstrated that *ca.* 75% of *Cryptosporidium parvum* oocysts and the cysts of *Giardia lamblia* bound to particles in secondary sewage effluent, which promoted sedimentation of the cysts/oocysts in line with the sedimentation velocity of the particles with which they were associated. Furthermore, *Cryptosporidium parvum* oocysts readily attach to suspended sediments in natural waters, enhancing their sedimentation in aquatic environments.[103]

Over 120 types of enteric virus are shed in human faeces alone, many of which enter the estuarine environment *via* sewage and septic tank discharges.[83] Viruses in the water column subsequently become associated with particulate material and settle to the benthos, where they accumulate as part of the surficial bed sediment[83] and much like bacterial pathogens, may be eroded and resuspended under turbulent hydrodynamic conditions and transported to areas of the estuary where public health may be impacted,[56] including beaches, bathing and shellfish harvesting areas. Sediments therefore also represent a significant reservoir of viruses, and several studies have reported that enterovirus concentrations in marine and estuarine sediments achieve up to 10 000-fold higher concentrations than the overlying water column.[83] Viruses readily attach to particulate material comprising both inorganic (clay, silt and sand) and organic particles (bacteria, algae, extracellular polymeric substances) and this process enhances the persistence of viruses in estuarine environments.[83] The exact mechanism of virus survival is unknown, but is likely to reflect a greater protection against inhibiting factors such as temperature, UV, bacterial/protozoan inhibition and salinity. Smith *et al.*[104] reported one of the first demonstrations of enhanced enteric virus persistence in estuarine microcosms where Echovirus, coxsackieviruses and poliovirus strains were added to estuarine microcosms with and without sediment. All viral types were detected for prolonged periods in sediments in relation to the viruses inoculated into water and the same effect was also observed when sewage effluent was added to the microcosms.[104]

5.2 Pathogen–floc interactions

In aquatic systems, association with flocs represents a medium for pathogen transport and survival and numbers of floc-associated *E. coli*, *Salmonella*, *Vibrio* spp. and coliforms are enriched several-fold when compared to the surrounding water,[55,56] representing a significant public health risk. Flocs are subject to a continuous flux, due to a breaking and rebuilding process that is governed by the composition, porosity, density, shape and particle size of the floc and their interaction with other environmental factors.[51] The bacterium–floc interaction is therefore a very transient process, and in constant interaction with the physical, chemical and biological conditions of its surroundings, all of which govern the settling velocity and potential resuspension and transport of floc-associated pathogens.[56] While we know that microorganisms can associate strongly with flocs, a full understanding of the factors involved in this process is poorly understood. For example, given the different external

cell surface composition and structure of different bacteria and viruses, it is likely that significant inter- and intra-species variability in floc binding strength exists. This binding is also likely to vary significantly with floc composition. For risk assessment purposes, it is important that these factors are better understood so that results obtained for one organism can be applied appropriately to other organisms.

Danovaro *et al.*[55] investigated the association of viral and bacterial pathogens with marine mucilage, demonstrating significant enrichment of mucilage-associated bacteria and viruses, when compared with the surrounding water. Furthermore, coliforms, *E. coli* and *Vibrio harveyi* were significantly enriched in the mucilage fraction, with the latter two bacterial species only detected in mucilage.[55] Droppo *et al.*[56] demonstrated that EPS produced by marine organisms can represent a significant component of flocculated material and plays a major role in the attachment and entrapment of pathogens within flocs. Analysis of culturable *E. coli* and *Salmonella* spp. in different river sediment compartments revealed that both bacteria were more abundant in the flocculated (suspended sediment) and surficial bed sediment fractions than in the water column and the greatest counts for both groups were found in the flocs. *E. coli* and *Salmonella* were detected in 94% and 89% of the floc samples, respectively, compared to 57% and 62% of the water samples.[56]

Shapiro *et al.*[105] performed *in vitro* microcosm studies to investigate the interaction of several zoonotic pathogens, including *Salmonella*, two protozoan pathogens (*Giardia lamblia* and *Cryptosporidium parvum*) and a virus surrogate (PP7) with macroaggreagates in fresh, estuarine and marine environments. Replicate microcosms were spiked with *Salmonella*, *Giardia, Cryptosporidium* or virus surrogate PP7 and either rolled (agitated) to promote aggregation of the pathogen and particulate material or unrolled, and the proportion of each pathogen associated with the aggregate rich or planktonic fraction of the microcosm was determined. These experiments suggested that pathogens were 2–4 orders of magnitude more concentrated in the aggregates when compared with estuarine and marine water with no aggregates. These studies demonstrate the strong link between pathogen survival and transport when associated with flocculated material. The association of pathogens with flocs increases their settling velocity, thus promoting their accumulation in the surficial bed sediment (SBS). However, SBS is often described as 'fluffy' due to variations in the strength and stability of the suspended material and the composition of the flocs; consequently, under certain hydrodynamic conditions, erosion of the SBS layer occurs, potentiating the remobilisation and transport of flocculated material and uptake by indigenous invertebrates such as shellfish, that represent important vectors of disease.

5.3 Pathogen–nutrient interactions

Nutrient availability is critical for pathogen growth and survival in any environment, and it is well established that nutrients in estuarine environments, and particularly organic rich sediments, can support the growth of *E. coli* and other enteric bacteria.[79,84,99,100] However, obtaining detailed information on the interaction of microbial pathogens with nutrients in estuarine environments is complicated due to the difficulties in separating out the effect of the numerous biotic and abiotic factors that affect pathogen survival in estuarine environments. Several studies report the growth of FB inoculated into sterilised microcosms where predation by protozoa has been eliminated, demonstrating the ability of pathogens to utilise estuarine nutrients.[79,84,99,100,106] Furthermore, the metabolism of sediment-associated nutrients by faecal bacteria in marine[82] and freshwater sediments has also been reported.[82,106] The aforementioned factors have obvious implications for the link between eutrophication/nutrient fluxes and the ecology of microbial pathogens in the freshwater–marine continuum.[107] However, *in situ* measurements and microcosm studies using unsterilised estuarine material typically demonstrate a net FB decay, suggesting that either protozoan predation masks pathogen growth *in situ*, or that detected pathogens represent those that are able to persist and avoid predation.[82,89,90,99,106]

A further complication in understanding the fate of FB such as *E. coli* in estuarine environments is their ability to enter a viable but non-culturable (VBNC) state. *E. coli* is an enteric bacterial species that typically resides in the intestine of mammals, where constant warm temperatures and a regular supply of nutrients (amino acids and sugars) support their growth and survival.[108] Excretion from the mammalian host results in the environmental deposition of *E. coli* cells, which experience numerous environmental stressors that include suboptimal temperature, osmotic stress, nutrient limitation, predation and competition for resources with indigenous niche-specialised microorganisms.[109] It has been proposed that certain bacteria, including *E. coli* and *Salmonella* spp. adopt a survival strategy by entering the VBNC state, which enables them to retain metabolic function whilst becoming intractable to cultivation on microbiological media. On return to favourable environmental conditions, it is proposed that cells may resuscitate from the VBNC state, retaining the ability to grow and potentiate infection; reviewed by Oliver.[110] The VBNC state has obvious implications for bathing and shellfish water quality testing that relies on enumeration by microbial culture counts that would fail to detect VBNC cells. However, with the exception of temperature, for which the resuscitation of VBNC *Vibrio vulnificus* has been shown to occur when temperature increases[111–113] the precise environmental factors that promote resuscitation from the VBNC state are barely understood. This is largely due to difficulties in conclusively distinguishing between 'truly' resuscitated cells and culturable cells that are derived from the regrowth of a small population of culturable cells that were present in the initial VBNC population and evaded detection.[110,114]

The fluxes of macronutrients from catchment to coast may therefore impact upon the resuscitation of microbial pathogens from the VBNC state, particularly when storm events and point and diffuse sources of nutrients enter estuarine environments, where they might stimulate pathogen growth. This is currently an area of research that requires attention, as it may have a significant impact on the fate of pathogenic organisms. The reader is referred to the following review of Oliver[109] for more information on the VBNC state in bacteria.

Nutrients are unlikely to promote direct metabolic effects on protozoan cysts (*e.g.* of *Giardia duodenalis)* and oocysts (*e.g.* those of *Cryptosporidium* spp. and *Toxoplama gondii*). However, the ionic composition of the surrounding water/sediment and the presence of other organic compounds affect the physico-chemical properties of protozoan cysts/oocysts, and specifically the charge and hydrophobicity of the parasites' surfaces. Consequently, these factors may either positively or negatively impact upon particle association, with differing implications for the survival and transport of protozoan cysts/oocysts in aquatic environments.[101]

Viruses are obligate intracellular parasites and are not living entities; consequently, they are not directly involved in nutrient metabolism. However, it is likely that enhanced nutrient concentrations would indirectly promote viral persistence by promoting microbial biofilm formation, EPS production and flocculation *in situ*, which would in turn promote the sorption of viruses to flocculated material and their enrichment in the sediment. Shapiro *et al.*[105] demonstrated a three order of magnitude enhancement in the association of virus surrogate PP7 with macroaggreagates when agitated in a micrososm, demonstrating that although flocs represent a passive substrate for viral attachment and persistence, nutrient interactions that promote flocculation would ultimately enhance virus attachment and promote survival. Further work is necessary to disentangle the interactions between allochthonous pathogens and estuarine nutrients, particularly with respect to the impact of enhanced nutrient deposition due to storm events, waste discharges and eutrophication.

The complex physical, biological and chemical interactions of bacterial and viral pathogens in estuarine environments pose a challenge when identifying the leading factors affecting pathogen survival. However, evidence suggests that sediment particle size and distribution has a significant impact on the spatial variation and persistence of human pathogenic bacteria and viruses within estuarine environments. Anthropogenic disturbance and hydrodynamic processes such as wave action and tides can re-suspend sediments back into the water column contaminating the surrounding area significantly impacting microbial water quality.

6. Impacts on shellfish waters

We have discussed the dynamic interactions between pathogens, nutrients and flocs but we must also consider how they may come to affect shellfish waters. As mentioned in section 3, there is, under normal riverflow conditions, a retention of particles within the estuary as a result of sediment pumping. Storm events alter this scenario by discharging a larger volume of freshwater than is typical. The salinity intrusion and ETM are pushed downstream within a few hours of the event but further adjustments (such as to the flocculation and SPM fluxes) are likely to take several days.[115] An increased volume of river water coming down the system is likely to increase water velocities on the ebbing tide (usually the smaller currents under typical conditions in a flood dominant system) which may cause the critical bed shear stress to be attained and sediment to be resuspended. In this case, material typically deposited during "normal" conditions, and that eroded from the bed, could be washed out of the system and transferred down the estuary and potentially out to sea. Additionally, enhanced winds may inject additional turbulence and cause erosion. The result of such a storm event would be to force all the material in the ETM, consisting of the aforementioned flocs, nutrients and other particles, downstream with a distance depending on the magnitude of the event. In their new locale, the flocs are once again subject to the same biogeochemical and physical processes as in their initial estuarine position, causing them to further flocculate, settle out of suspension or break apart.

7. Conclusions

Estuaries are highly productive biological systems and intrinsically offer a diverse range of ecosystem services and are associated with a diverse and high population density of organisms. Consequently, anthropogenic impacts associated with land management, industry and waste generation can have profound effects on ecosystem functioning in the downstream catchment and associated coastal zone. Transfer of macronutrients, sediment, and microbial pollutants (derived from human and animal waste) from land to sea are thought to have significant impacts upon estuarine environments, however, our knowledge and understanding of the interactions between these factors and their influence on estuarine processes and public health are poorly understood. This is particularly pertinent with respect to extreme events (*e.g.* periods of storm flow or extreme low flow followed by rain events), which have the potential to increase hydrodynamic flow and the input of nutrients, sediment and untreated faecal contents into estuarine systems. It is well established that human microbial pathogens preferentially attach to particulate material, which potentiates their persistence and downward flux into the sediments. At a much later date, these can become re-suspended and transported from the estuary to high risk zones (*e.g.* recreational beaches and bathing waters) and shellfish harvesting areas providing an opportunity for the pathogens to be reintroduced back into the human population. It is therefore critical that the interactions between pathogens, macronutrients and flocculated material and the physical, biological and chemical processes that underpin these interactions are better understood in order to advise on public health risk, mitigation strategies and in the modelling of predicted pathogen behaviour under future environmental scenarios.

Future research priorities

It is clear from this review that considerable uncertainty surrounds the flow of microbial contaminants from agricultural catchments through to the coastal zone. This lack of fundamental knowledge is limiting the implementation of effective mitigation measures and the formulation of robust policies and legislation to protect human health and the wider environment. Additional research is therefore required to disentangle the complexity of bacterial, protozoan and particularly viral

interactions within water, sediments, nutrients and flocs along freshwater–saline gradients. Further, there is a critical need to understand how pathogen dynamics will change in response to environmental perturbation such as long term climate change and the increased prevalence of extreme weather events. In some situations, these changes in climate are likely to exacerbate the risk of human exposure to bathing water- and food-borne pathogens. In the next few decades, it is likely that we will also see shifts in land use in response to the drive towards more sustainable agricultural systems (*i.e.* sustainable intensification). This may bring new challenges in preventing the flow of animal-derived pathogens into coastal zones. An improvement in fundamental knowledge and the creation of new modelling tools could allow us to explore and predict the impacts of these changes in land use on pathogen behavior prior to their implementation. Current monitoring of potential harmful organisms within freshwaters and the marine zone is extremely limited both in space, time and organism scope. However, the emergence of new high-throughput molecular platforms and sensor devices makes the possibility of real-time detection almost a reality and research in this area should be prioritized. We also need to move away from traditional approaches which simply rely on indicator bacteria (*e.g. E. coli*/coliforms) towards more inclusive detection approaches targeted towards a suite of known pathogenic organisms or virulence genes allowing risk assessments to become much more holistic and informative. Future research should also be directed towards greater surveillance of novel pathogens in freshwater and marine environments to allow health protection agencies to better prepared against the emergence of new pathogenic organisms. In terms of the ecology of viral pathogens, the effects of environmental factors on their human infectivity remains poorly understood. New *in vitro* viral infection systems are clearly needed to take this work further. In addition, the importance of viable but non-culturable bacteria that may retain the potential to infect humans after residence in the freshwater–marine continuum needs greater clarification. Future research programmes will require, and benefit, from multidisciplinary multi-partner and interagency working, including stakeholders involved in wastewater treatment and riverine, estuarine and coastal environments looking at reservoirs of microbial pathogens, their suspension and potential reactivation to determine mitigation actions and improve water quality and food security.

Acknowledgements

This research was supported by the UK Natural Environment Research Council under the Macronutrients Programme from a NERC grant: NE/J011967/1: The Multi-Scale Response of Water Quality, Biodiversity and Carbon Sequestration to Coupled Macronutrient Cycling from Source to Sea'. This research was also supported by a Knowledge Economy Skills Scholarship (KESS) awarded to T.L.P, funded *via* the European Social Fund (ESF) through the European Union's Convergence program administered by the Welsh Government in association with Dŵr Cymru Cyf/Welsh Water Ltd.

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