Title: High prevalence of *Legionella* in water systems of merchant vessels docking at UK ports, 2013 to 2016

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Short title: *Legionella* contamination in non-passenger merchant vessels

Introduction

Throughout history ships have played a major part in the global transmission of human disease. Today the international shipping industry is huge with more than 100,000 sea-going merchant vessels in operation with an average age of 22 years. Approximately 55,000 of these represent cargo carrying (non-passenger merchant vessels, NPMVs) with an average age of 19 years (1).

The provision of safe, potable water on board merchant ships is critical for the health of passengers and crew alike. This can be particularly challenging as ship water systems can be complex and contamination can occur at multiple points during supply, bunkering, treatment, storage, distribution and use. In 2001 the World Health Organisation (WHO) attributed one fifth of all ship-associated outbreaks to contaminated water or ice (2) and a number of these incidents are well documented (3-5). The revised edition of the WHO guidelines (6) recognises that the risk of waterborne outbreaks on board ships can be mitigated through the appropriate handling of potable water and the maintenance of storage and distribution systems. One highly recommended approach for achieving this is to implement a water safety plan (WSP), encompassing the principles of Hazard Analysis and Critical Control Points (HACCP). Such plans are based on a three stage model encompassing a risk assessment, implementation and monitoring of control measures and establishment of good management procedures (7).

Legionellae and their protozoan hosts are ubiquitous in freshwater sources but thrive in manmade water systems operating between 20 and 45 degrees Celsius. They are the causative agents of two distinct clinical syndromes, Legionnaires' disease, a severe form of pneumonia (8) and Pontiac fever, a milder self-limiting illness (9). Infection typically follows the inhalation of contaminated aerosols from water sources such as cooling towers, spa pools and hot and cold water systems. Numerous other potential sources have been described (10-16). Worldwide the incidence of Legionnaires' disease is increasing (17) with the highest number of reported cases ever in Europe in 2014 (17). The WHO provides guidance (6, 18) on managing the risks associated with *Legionella* on board merchant vessels. Despite the existence of this advice and other country-specific guidance (19, 20) several studies have shown a high prevalence of *Legionella* contamination in passenger

The control of *Legionella* in water systems in the UK is supported by the Health and Safety Executive (HSE) Approved Code of Practice (ACoP) L8 and Health and Safety Guidance HSG 274 (20). This guidance provides action and alert levels (table 1) which are used by Port Health Officers to interpret results of *Legionella* testing and recommend remedial actions.

This retrospective study analysed microbiological test results of routine potable water samples collected from merchant vessels docked at UK Ports between 2013 and 2016. To our knowledge this is the first report on the prevalence of *Legionella* contamination in NPMVs.

Materials and Methods

Sample collection

Samples were collected between April 2013 and February 2016 by appropriately trained officers during routine inspections by Port Health Authorities. Vessels were docked in the Ports of Belfast, Bristol, Falmouth, Fowey, Manchester, Plymouth, Southampton and Weymouth. For *Legionella* analysis one litre samples were obtained in accordance with BS7592:2008 using sterile plastic bottles. All samples were taken without flushing. Showers were chosen as a representative point of the water system for *Legionella* sampling due to ease of access, risk presented to crew/passengers and because they tend to represent a mixture of both the hot and cold water systems. Samples from showers were taken using a sterile bag wrapped around the shower head to funnel water into the sample bottle while minimising aerosol generation. To monitor the general microbiological quality of potable water, samples were also taken for aerobic colony count (ACC) coliforms/E.coli and enterococci using 500 ml bottles in accordance with Health Protection Agency (now Public Health England) guidelines (34). All sampling bottles contained 20mg/L sodium thiosulphate to neutralise oxidising biocides. Details including the ship's name, registration age and size (gross tonnage) were recorded. All samples were transported to the laboratory under

enterococci were confirmed by the aesculin hydrolysis test. ACC coliforms, E. coli and enterococci test results were interpreted with reference to published HPA guidelines (34). Results were deemed unsatisfactory if greater than the action limits of 1000 CFU/ml and 1 CFU/100 ml for ACC and enterococci respectively. *Legionella* results were interpreted according to HSE guidelines (20) (Table 1).

Sample analysis by real-time PCR

From June 2014 until March 2015 *Legionella* samples were also examined by quantitative polymerase chain reaction (qPCR) as part of an evaluation of qPCR for all water samples submitted to our laboratories. DNA was extracted from sample concentrates and a duplex real-time PCR for *Legionella* spp. and *L. pneumophila* performed as previously described (38) with the following modifications: TaqMan® Fast Environmental Master Mix Beads (Life Technologies, Carlsbad, USA) incorporating a VIC 3' labelled internal positive control were used for all real-time PCR reactions. Samples were processed in an ABI 7500 FAST thermocycler (Life Technologies). Thermal reaction conditions were 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. A positive control (*L. pneumophila* sg1 NCTC 12821) and a non-template control (PCR grade water, Millipore, Watford, UK) were included in all qPCR assays. Five calibration standards (SRM-LEGDNA-01) [LGC Standards, Teddington, UK] ranging from 10 to $1x10^5$ genome units (GU)/reaction were used to generate standard curves for qPCR quantification. The limits of detection (LOD) and quantification (LOQ) of the qPCR assay were 150 and 300 genome units (GU)/L. Real-time PCR data was analysed using ABI 7500 v2.3 Software (Life Technologies).

Sequence based typing

Select *L. pneumophila* sg-1 isolates were subjected to sequence based typing (SBT) according to an internationally validated method (39-41). SBT results were analysed using the online Sequence Quality Tool (www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi) to obtain allelic profiles and sequence types (STs). Alleles that could not be assessed by SBT were marked as '0'.

followed by chemical/oil product tankers (19.3%, 31/160). Two of 10 (20%) cruise ships and 2/11 (18.2%) passenger vessels also failed on at least one sample. A total of 422/1027 (41%) results were greater than the action limit of 1000 CFU/ml. The proportion of actionable samples was slightly higher at 37°C than at 22^0C (Table 2) although this was not significant (p= 0.46, Chi squared test). ACC results ranged between 0 and $2.08x10^5$ CFU/ml (median = 37 CFU/ml) and 0 to $9.6x10^5$ CFU/ml (median = 84 CFU/ml) for 37°C and 22^0C respectively. Actionable levels at 37^0C (indicating a possible deterioration in potable water quality (42)) were most frequent in drinking water taps (21.1%, 87/412) followed by galley taps (19.2%, 98/512). Almost one third (31.8%, 14/44) of drinking water dispensers also returned actionable levels.

For Coliforms 40/985 (4.2%) were unsatisfactory (>1 CFU/100ml), representing 34 individual vessels (Table 2). Cargo ships had the highest rate of failure for coliforms (50% of positive vessels) followed by chemical/oil product tankers (20%). No passenger vessels experienced failures. Coliforms ranged between 1 and >201 MPN/100ml. The majority of positive samples were obtained from galley taps (32/40, 78%) followed by other potable taps (7/40, 17.1%) and one drinking water dispenser (2.4%). Only one sample from a galley tap (0.1%) was positive for *E.coli*. For enterococci, the number of vessels with unsatisfactory samples (>1 CFU/100ml) was four (0.9%). Three galley taps and one other potable outlet were positive with results between 1 and 7 CFU/100ml. (Table 2).

Vessels positive for Coliforms, E.coli or enterococci had a mean age of 17.8 years (95% CI 15 to 22) which was not significantly higher (p = 0.4, unpaired *t* test) than the mean of all the vessels sampled. Fourteen of the 34 positive vessels were also associated with actionable ACC results.

Legionella

Eight hundred and three *Legionella* samples were analysed from 360 vessels with an average age of. One hundred and ninety eight vessels were sampled at least twice either on the same day or on separate port dockings. The majority of samples (589/803, 73.3%) were obtained from crew and cabin showers, 13.6% from other potable taps, 9% from hospital

Sequence-based typing

To gain an insight into the distribution of *L. pneumophila* sg1 sequence types, available isolates (n = 17) obtained from 17 individual vessels (x cargo, x research, x cruise, x chemical/oil products tanker, X RFA) between 2013 and 2016 were analysed according to the international sequence-based typing scheme. Complete or partial profiles were obtained for all isolates.

Discussion

In the UK, Port Health Authorities (PHAs) are responsible for inspecting and sampling potable water systems on board merchant vessels. Between 2013 and 2016, X water samples from vessels docking in X UK ports were collected by PHA officers and analysed for multiple microbiological parameters including *Legionella*.

Four percent of samples were positive for coliforms and less than 1% for enterococci and *E. coli*, representing an improvement on a previous study (31) and indicating that the quality of potable water in terms of potential faecal contamination has improved. All Port Health Authorities involved in this study undertook ACC sampling. Forty percent of vessels had one or more ACC results above the action limit indicating a potential deterioration in potable water quality. This is very similar to a previous study conducted on ships in UK Ports in 2008 (31). According to HPA guidelines, ACC results >1000 CFU/ml would necessitate a hyper-chlorination and refilling of the potable water supply. Whilst ACC testing is quick and can provide actionable results to Port Health Officers while a vessel is still docked, interpreting results is difficult particularly when considering an individual sample result in the absence of ongoing trend data. It is recognised that bacterial numbers will gradually increase in stored water, and high levels of ACC do not necessarily correlate to a public health risk (43). We agree with Grenfell *et al* that ACC sampling for vessels should be reconsidered and public health resources better focused on monitoring for faecal indicators (43), and from the data presented in this study, *Legionella*. ACC testing will remain a useful indicator of disinfection efficacy and can be used for trend analysis to monitor water quality over time.

for SBT nonetheless this is an interesting finding and suggests that a large proportion of *L. pneumophila* sg1 positive vessels are colonised with STs of clinical importance. To investigate the distribution of STs further it would be advantageous to sequence type more isolates including multiple isolates from the same vessel.

The majority of positive vessels however were colonised with L. pneumophila sg-2-14 obtained from ship waters Discuss the significance of *Legionella* sg2-14 isolation i.e. have been outbreaks on ships from these sgs before, could also indicate potential for sg1 colonisation etc.

The use of qPCR provided a more sensitive technique compared to culture, identifying an additional 41 *Legionella* positive samples that were negative by culture. In total a further 16 vessels were identified to be *Legionella* positive by qPCR alone. The qPCR positivity rate for ship waters was higher than any other water system (cooling towers, hot and cold, spa pools) examined by our laboratory in the same period (submitted). Reasons for the discrepancies between culture and qPCR are well documented (refs) and include the detection of *Legionella* species that don't grow (or are overgrown by background flora) under standard laboratory conditions as well as dead, injured or viable but non-culturable cells. Despite the potential to detect dead cells the relatively high GU/L results for the majority of these vessels (median $2.05x10^4$ for *Legionella* spp.) suggests ongoing contamination that should warrant further investigation. Quantitative PCR has previously been used as an effective screening tool for environmental samples (38, 47). The sensitivity and speed of qPCR analysis compared to culture suggests that it could be used as a more rapid indication of *Legionella* contamination in ship waters. The high negative predictive value could allow negative samples to be screened out rapidly. The detection of high concentrations of *Legionella* DNA could be used as a platform on which PHA's can recommend remedial action in a timely manner not possible with culture.

For all the *Legionella* samples taken, X% of these would require action to be taken based on UK *Legionella* legislation (20). At the very least this action should include cleaning, flushing and resampling of the implicated outlet as well as a review of the Legionella risk assessment. It can be difficult to enforce this action as UK Port Health Authorities have

Coliforms, *E.coli* and enterococci results were encouraging and indicated the general quality of potable water on board vessels had improved in recent years. However, it is clear from data presented in this study that further actions are required by operators and crews of merchant vessels to control the risk from *Legionella*. Any WSP on board ships must contain provisions for the control of *Legionella* and there are several good guidance documents available to help ship operators. However, these documents are largely aimed at passenger vessels. Whilst these carry the greatest risk for members of the public NPMVs contribute more than half of the global sea-going fleet and are manned by well over a million seafarers. We therefore recommended that additional guidelines specific for NPMVs be developed with a strong emphasis on the control of *Legionella*. Such guidelines should also incorporate a recommendation to cease one-off ACC sampling and focus resources on more useful indicator organisms.

This study is the first to report on the presence and prevalence of *Legionella* in NPMVs. Vessels were found to be frequently contaminated with *Legionella* and a large proportion of results were greater than the upper action limit dictated by UK legislation. Sequence-based typing indicated that vessels are contaminated with *L. pneumophila* sg-1 STs of clinical significance. This presents a risk of infection to merchant seafarers and raises significant concerns about the water quality and management of *Legionella* on board NPMVs. These findings reinforce the need for vessel operators to establish a robust *Legionella* control scheme as part of a Water Safety Plan based on the model laid out by the WHO. Current guidelines should be revised to specifically encompass NPMVs. Vessels take on water from ports throughout the world. The majority of vessels positive for *Legionella* in this study undertook international routes and could therefore represent an efficient mechanism for the spread of *Legionella* isolates (including those of clinical significance) around the world, a possibility that warrants further research. This study provides preliminary data that could be used to support a future, industry wide evaluation of Legionella contamination and control on NPMVs.

Anecdotal - control measures in place (nominal) however understanding of why + sampling to ascertain efficacy patchy

MLC?

	ACC 22°C (68 ± 4 hr)	ACC 37°C (44 ± 4 hr)	Enterococci	Coliforms	*E. coli*
Samples > action level*	204/1023	218/1027	4/985	40/985	1/985
% of samples	19.9	21.2	0.4	4.0	0.1
% of vessels with samples >action levels	38.8	38.8	0.9	7.8	0.2

Table 2: Samples positive for aerobic colony count (ACC), coliforms, *E. coli* and enterococci with respect to published guidelines. *Action levels are >1000 CFU/ml for ACC and >100 CFU/100ml coliforms, *E.coli* and enterococci.

Sampling point	No. of samples	*Legionella* pos. by culture (%)	Lower action limit >100 CFU/L (%)	Upper action limit >1000 CFU/L (%)
Crew shower	587	272 (46.3)	103 (17.5)	161 (27.4)
Tap	111	62 (55.8)	19 (17.1)	37 (33.3)
Hospital shower	73	33 (45.2)	11 (15.0)	22 (30.1)
Other	32	6 (20)	0 (0.0)	4 (13.3)
Total	803	373 (46.5)	133 (16.5)	224 (27.9)
% of vessels		58.3	50.2	43.3

Table 3: *Legionella* culture results according to UK action and alert criteria (20). The percentage of vessels positive for *Legionella* above the lower and upper action limits is indicated.

Figures

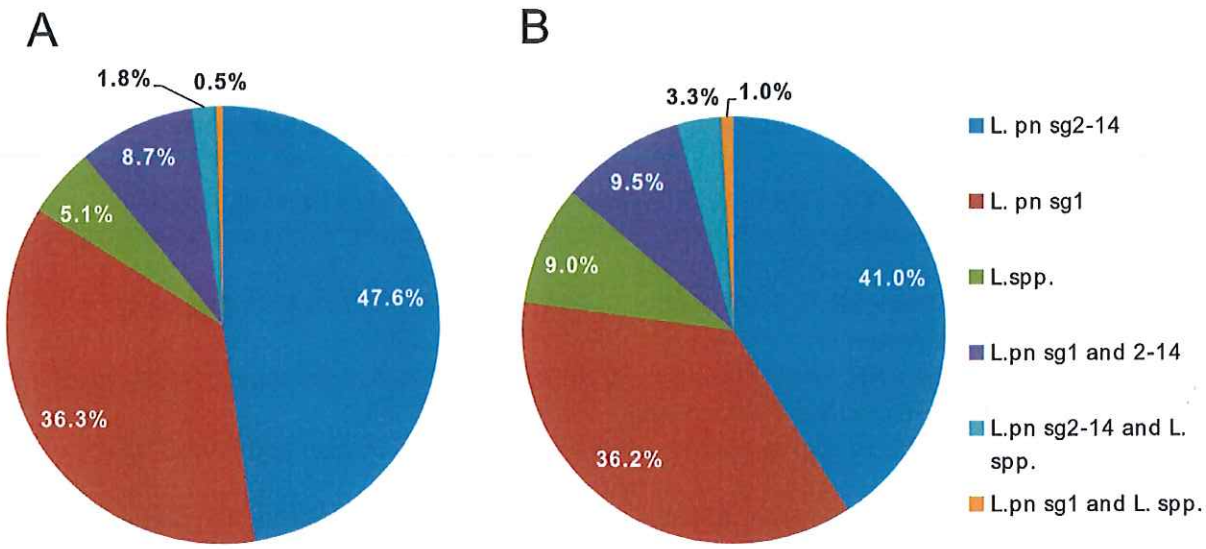

Figure 1: (A) Distribution of *Legionella* by type in positive samples (n = 278). (B) Proportion of vessels positive for each *Legionella* type.

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