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**Detection of OXA-48-like-producing *Enterobacterales* in Irish recreational water**

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## Abstract

The rapid dissemination of carbapenemase-producing *Enterobacterales* (CPE) is a major public health concern. The role that the aquatic environment plays in this dissemination is underexplored. This study aimed to examine seawater as a reservoir for CPE. Seawater sampling took place at a bathing site throughout the 2017 bathing season. Each 30 L sample (n=6) was filtered using the CapE filtration system. Wastewater samples (200 mL) (pre-treatment (n=3) and post-treatment (n=3)) were obtained from a nearby secondary wastewater treatment plant, during the same time period. All samples were examined for CPE. Whole genome sequencing of confirmed CPE was carried out using Illumina sequencing. Isolate genomes were hosted in corresponding BIGSdb databases and analyses were performed using multiple web-based tools. CPE was detected in 2/6 seawater samples. It was not detected in any wastewater samples. OXA-48-like-producing ST131 *Escherichia coli* (Ec\_BM707) was isolated from a seawater sample collected in May 2017 and OXA-48-like-producing ST101 *Klebsiella pneumoniae* (Kp\_BM758) was isolated from a seawater sample collected in August 2017. The genomes of the environmental isolates were compared to a collection of previously described Irish clinical OXA-48-like-producing *Enterobacterales* (n=105). Ec\_BM707 and Kp\_BM758 harboured *bla*<sub>OXA-48</sub> on similar mobile genetic elements to those identified in the clinical collection (pOXA-48 fragment in Ec\_BM707 and IncL(pOXA-48) plasmid in Kp\_BM758). Genetic similarities were observed between Ec\_BM707 and several of the clinical ST131 *E. coli*, with allele matches at up to 98.2% of 2513 core genome multilocus sequence type (cgMLST) loci. In contrast, Kp\_BM758 and the 34 clinical *K. pneumoniae* were genetically distant. The source of the CPE at this site was not identified. The detection of OXA-48-like-producing ST131 *E. coli* and OXA-48-like-producing ST101 *K. pneumoniae* in Irish recreational water is a concern. The potential for contamination of the aquatic environment to contribute to dissemination of CPE in Europe warrants further study.

**Keywords:** antimicrobial resistance; carbapenemase-producing *Enterobacterales*; recreational water

## 1. Background

Antimicrobial resistance (AMR) is recognised as one of the biggest threats to public health and global efforts are being made to control this escalating problem (WHO, 2015). Carbapenemase-producing *Enterobacterales* (CPE) are one of the antimicrobial resistant organisms of greatest concern (The Department of Health, 2017). Infections caused by CPE are associated with increased mortality rates and higher healthcare costs (Falagas et al., 2014) (Borer et al., 2009). In addition to being resistant to the carbapenems, these organisms are frequently co-resistant to several other antimicrobial classes due to the presence of multiple resistance genes (Nordmann et al., 2011).

There are many different types of carbapenemases, the most common of which are Oxacillinase-48-like (OXA-48-like), *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-beta-lactamase (NDM) and Verona integron-encoded metallo-beta-lactamase (VIM) (Brolund et al., 2019). The prevalence of each varies between geographical regions (Brolund et al., 2019). OXA-48-like is the most common carbapenemase identified in Ireland, with 394 (73.5%) of 536 clinical cases of CPE in 2018 confirmed as OXA-48-like producers (HPSC, 2019). The *bla*<sub>OXA-48</sub> encoding gene is most commonly associated with an IncL(pOXA-48) plasmid (within transposon Tn1999), and this plasmid has been reported in multiple species of the *Enterobacterales* (Skalova et al., 2017) (Poirel et al., 2012). There have also been several recent reports of the detection of a fragment of pOXA-48 (containing *bla*<sub>OXA-48</sub>) chromosomally integrated into strains of *Escherichia coli* and *Klebsiella pneumoniae* (Skalova et al., 2017) (Beyrouthy et al., 2014) (Turton et al., 2016).

The 'One Health' approach recognises that human, animal and environmental health are inextricably linked (WHO, 2015). The environment remains the most under investigated

aspect of the One Health triad and the role that it plays in the dissemination of AMR is not well understood (Essack, 2018). A number of studies have reported the detection of CPE in freshwater bodies in Europe, with several types of carbapenemases including OXA-48-like, KPC, NDM, VIM and IMP identified (Khan et al., 2018) (Zurfluh et al., 2013) (Kieffer et al., 2016) (Kittinger et al., 2016) (Zarfel et al., 2017). We recently reported the first detection of NDM-producing *Enterobacterales* in European seawaters, with untreated human sewage discharges identified as the source of CPE (Mahon et al., 2017). The aim of our current study was to investigate if seawaters not receiving any apparent raw sewage discharges may also act as a reservoir of CPE. We also aimed to establish if CPE present in the aquatic environment was similar to CPE present in humans in Ireland during the same time period.

## 2. Methods

### 2.1 Sampling sites and sample collection

Seawater from a bathing site located in the West of Ireland was assessed for the presence of CPE, throughout the 2017 bathing season (Fig. 1, Supplementary File 1). A total of six 30 L samples were collected between May and September 2017. Samples were collected in sterile 5 L containers (x6) and were obtained approximately 30 cm below the water's surface, in water at least one metre in depth. The bathing site is located within close proximity to a secondary waste water treatment plant (SWWTP) (Fig. 1). To the authors knowledge this is the only point source release in this area. Samples (200 mL) of influent (pre-treatment, n=3) and effluent (post-treatment, n=3) were collected from this treatment plant during the same time period. The sewage samples were collected in sterile 1 L glass bottles. All seawater and sewage samples were subsequently transported to the laboratory and processed within 24 hours of collection. Samples which were not processed immediately were stored in the dark at 2-8°C until processing took place. The rainfall measurements for the 24 hours preceding the seawater sampling dates were obtained from the Informatics

Research Unit for Sustainable Engineering (IRUSE) (<http://www.iruse.ie/>). This data was examined to determine if stormwater overflows could potentially be associated with the presence of any CPE detected in the samples taken on the corresponding dates.

## 2.2 Processing of samples and preliminary characterisation of CPE

Following collection, each 30 L seawater sample was filtered through a 0.45 µm membrane filter (Millipore) using the CapE large volume filtration system (Morris et al., 2016). This filter was then placed into 100 mL of buffered peptone water and incubated overnight at 42°C. The enrichments were subsequently sub-cultured onto Brilliance CRE agar plates (Oxoid) using a sterile cotton swab. The pre-treatment and post-treatment sewage samples were cultured directly onto this selective agar. The plates were then incubated overnight at 37°C. Matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry (Bruker; Biotyper version 4.1) was used to identify suspect *Enterobacterales* to species level (in line with the manufacturer's guidelines). Isolates identified as members of the *Enterobacterales* family were stored for further characterisation. Antimicrobial susceptibility profiles were generated for each isolate by performing susceptibility testing against 14 antimicrobials (listed in Table 1), via the disk diffusion method (European Committee on Antimicrobial Susceptibility Testing guidelines) (EUCAST, 2017). Suspect CPE were screened for carbapenemase encoding genes by real-time PCR (*bla*<sub>OXA-48</sub>, *bla*<sub>KPC</sub> and *bla*<sub>NDM</sub> as previously described (Swayne et al., 2011) (CDC, 2011) and *bla*<sub>VIM</sub> and *bla*<sub>IMP</sub> using an unpublished duplex assay developed by the National Carbapenemase-Producing *Enterobacterales* Reference Laboratory Services (NCPERLS)). Appropriate ATCC/NCTC strains were used for quality control purposes.

## 2.3 Whole genome sequencing analysis

Total genomic DNA was extracted from confirmed CPE using the Qiagen EZ1 Advanced XL automated extraction system. Paired-end short-read sequencing of prepared DNA was performed using an Illumina HiSeq platform. Following quality checks, *de novo* assembly of short reads was carried out using the VelvetOptimiser algorithm (Zerbino, 2010). The Center

for Genomic Epidemiology web tools (<http://www.genomicepidemiology.org/>) were used to identify AMR determinants and plasmid replicon content.

### **2.3.1 Comparison of environmental OXA-48-like-producing *Enterobacterales* to clinical CPE**

Further analysis was carried out to determine if the strains/mobile genetic elements identified in this study were similar to those present in the human population in Ireland during the same time period. The Irish NCPERLS provided access to the genomes of 105 OXA-48-like-producing *Enterobacterales* for this purpose. This clinical collection originated from healthcare facilities throughout Ireland (all isolated during 2016/2017) and have previously been described (Brehony et al., 2019). Genome analysis tools within the *Klebsiella* (<http://bigsdb.pasteur.fr/klebsiella/>) and *Escherichia* (<https://pubmlst.org/escherichia/>) BIGSdb databases were used to examine the genetic relatedness of the two collections. Core genome multilocus sequence type (cgMLST) comparisons at 2513 loci for *E. coli* and 629 for *K. pneumoniae* were visualized using minimum-spanning trees. This was performed with genomes within the same ST and was generated using the GrapeTree tool in BIGSdb (Zhou et al., 2018). pOXA-48 plasmid comparisons were performed by comparing isolates at 71 loci (present in the pOXA-48 plasmid (JN626286)), as previously described (Brehony et al., 2019).

## **3. Results:**

### **3.1 Detection of CPE in seawater samples**

CPE was detected in two of six seawater samples examined. OXA-48-like-producing *E. coli* (isolate ID: Ec\_BM707) was detected in a sample collected on May 29th, 2017, while OXA-48-like-producing *K. pneumoniae* (isolate ID: Kp\_BM758) was detected in a sample collected on August 14th, 2017. According to data from IRUSE, the daily rainfall measurements in this region during the sampling time period (27<sup>th</sup> May to 11<sup>th</sup> September)

ranged from 0 mm to 49 mm (average daily rainfall of 4.7 mm). There was a total of 0 mm rainfall recorded in the 24 hours preceding May 29th, and 8 mm in the 24 hours preceding August 14<sup>th</sup>. Samples of influent and effluent were collected from a nearby SWWTP during the same time period and examined for CPE. CPE was not detected in any of these influent (n=3) or effluent (n=3) samples.

### 3.2 Characterisation of OXA-48-like-producing *E. coli* (Ec\_BM707)

Ec\_BM707 was non-susceptible to 8/14 of the antimicrobials tested (Table 1). It remained susceptible to cefoxitin, chloramphenicol, gentamicin, kanamycin, tetracycline, trimethoprim. *In silico* analysis identified Ec\_BM707 as a member of ST131. In addition to *bla*<sub>OXA-48</sub>, it co-harboured *bla*<sub>CTX-M-174</sub> and *mdf(A)*. A total of four plasmid replicon types were detected: Col156, IncFIA, IncFIB and IncFII. Ec\_BM707 did not harbour an IncL(pOXA-48) plasmid, but analysis indicated that *bla*<sub>OXA-48</sub> was harboured on a pOXA-48 fragment, as 29/71 pOXA-48-like genes were detected in this isolate. The location of this fragment within the genome is unknown. Ec\_BM707 was compared to a collection of previously described clinical OXA-48-like-producing *Enterobacterales* (n=105) (Brehony et al., 2019). Nine of these clinical isolates (eight *E. coli* and one *K. pneumoniae*) carried *bla*<sub>OXA-48</sub> on a similar pOXA-48-like fragment (the same 29 genes were identified in these isolates). However, the alleles present in Ec\_BM707 differed to those found within the nine clinical isolates, with genetic diversity varying at between 14% and 21% of the 29 loci. Of the 55 *E. coli* within the clinical collection, nine belonged to ST131. A cgMLST comparison of Ec\_BM707 to the nine ST131 isolates at 2513 loci showed that the closest related clinical isolate (ERR2124250) had only 1.8% allele differences (46/2513) (Fig. 2). Of the nine clinical ST131, three (including ERR2124250) harboured *bla*<sub>OXA-48</sub> on a pOXA-48 fragment, with the remaining six harbouring the encoding gene on an IncL(pOXA-48) plasmid.

### 3.3. Characterisation of OXA-48-like-producing *K. pneumoniae* (Kp\_BM758)



Kp\_BM758 was non-susceptible to all 14 antimicrobials tested (Table 1). It was identified as ST101, and along with *bla*<sub>OXA-48</sub>, it co-harboured 15 additional resistance genes, including *bla*<sub>CTX-M-15</sub> (Table 1). Six plasmid replicon types were detected: Col440II, IncFIA, IncFIB, IncFII, IncL(pOXA-48) and IncR, with *bla*<sub>OXA-48</sub> carried on the IncL(pOXA-48) plasmid. Kp\_BM758 was compared to the same collection of 105 clinical OXA-48-like-producing *Enterobacterales*. Like Kp\_BM758, 89% (n=93) of the clinical isolates harboured *bla*<sub>OXA-48</sub> on an IncL(pOXA-48) plasmid. Kp\_BM758 differed at between 9% (n=6) and 14% (n=10) of the 71 pOXA-like loci, when compared to the IncL(pOXA-48) plasmids from those 93 isolates. Of the 34 *K. pneumoniae* within the clinical collection, none were ST101, and BM\_758 and the 34 isolates varied at between 72% (n=454) and 85% (n=533) of 629 cgMLST loci.

#### 4. Discussion

The dissemination of CPE poses a significant threat to public health. The potential reservoirs and dissemination routes of AMR are complex and are only partially understood. Studies indicate that many clinically relevant resistance genes, such as *bla*<sub>OXA-48</sub> and *bla*<sub>CTX-M</sub> originated in environmental bacteria, demonstrating that the environment can act as an ideal setting for the horizontal gene transfer of mobile genetic elements (Poirel et al., 2004) (Cantón et al., 2012). To date, many different types of carbapenemases have been isolated from freshwater sources (Khan et al., 2018) (Kittinger et al., 2016) (Kieffer et al., 2016) (Zurfluh et al., 2013) (Zarfel et al., 2017). This study investigated the presence of CPE in seawater samples collected at a bathing site during the 2017 bathing season. Over the course of the bathing season we detected two different species of *Enterobacterales* harbouring *bla*<sub>OXA-48</sub> (*E. coli* (Ec\_BM707) and *K. pneumoniae* (Ec\_BM758)).

In a previous study carried out in 2016-2017, we reported the first identification of CPE (NDM-producing *Enterobacterales*) in seawater in Europe (Mahon et al., 2017). The source of CPE at this site was identified as untreated human sewage, which was being discharged

nearby. In the present study there were no apparent sources of untreated human sewage discharges in this area. Rainfall levels in the 24 hours prior to sampling were low, which makes storm water overflows an unlikely source. CPE was not detected in any of the samples of treated wastewater effluent collected from a nearby SWWTP. However, this does not rule out treated effluent as a potential source of CPE at this site, as the effluent sample size was small ( $n=3$ ), the sample volume was much lower (200 mL) when compared to the bathing water sample volume (30 L) and the method applied (direct plating of sample onto selective agar) had limitations. In a recent publication we have shown that CPE are regularly present in untreated sewage that is processed in this SWWTP (Cahill et al., 2019). The last century has seen vast improvements in the infrastructure of WWTPs. However, WWTPs have not been specifically designed to combat AMR (Bürgmann et al., 2018), and multiple studies have detected AMR *Enterobacterales* including extended-spectrum  $\beta$ -lactamase-producing *Enterobacterales* and CPE, as well as their mobile genetic elements in effluent from WWTPs (Korzeniewska and Harnisz, 2013) (Müller et al., 2018) (Woodford et al., 2014).

A recent study (Brehony et al., 2019) characterised a collection of 105 OXA-48-like-producing *Enterobacterales* isolated from clinical specimens in Ireland during a similar time-period (2016/2017). High-resolution genomic analysis was used to investigate the genetic relatedness of environmental isolates Ec\_BM758 and Ec\_BM707 to this collection of clinical CPE. Along with the OXA-48-like gene, Ec\_BM707 harboured two additional resistance genes, including CTX-M-174. This CTX-M variant is relatively uncommon and was not identified among the collection of clinical CPE (Brehony et al., 2019). Ec\_BM707 belonged to ST131. This sequence type has contributed to the rapid global dissemination of *bla*<sub>CTX-M-15</sub> (Price et al., 2013) and is increasingly associated with OXA-48-like-producing *E. coli* (Agabou et al., 2014) (Dimou et al., 2012). ST131 was the most common sequence type identified among the *E. coli* within the Irish clinical collection (9/55). The nine ST131 *E. coli* originated from five different hospitals throughout Ireland showing that ST131 is widely

disseminated among OXA-48-like producing *E. coli* within the Irish health care setting (Brehony et al., 2019). A high level of genetic similarity among cgMLST genes was observed between Ec\_BM707 and several of the clinical ST131 isolates, indicating that they were closely related (Fig. 2). Like Ec\_BM707, 33% of the clinical ST131 *E. coli* harboured *bla*<sub>OXA-48</sub> on a pOXA-48 fragment. While chromosomal integration of this fragment type into ST131 *E. coli* has previously been reported (Beyrouthy et al., 2014), without the addition of long read sequencing, the exact location of this fragment within the genome of Ec\_BM707 is unknown.

Kp\_BM758 harboured 15 additional resistance genes, including five further beta-lactamase encoding genes, and these genes were similar to those reported in the clinical collection (Brehony et al., 2019). Kp\_BM758 was a member of ST101 which is considered a pandemic clone (Roe et al., 2019), and has previously been associated with outbreaks of OXA-48-like-producing *K. pneumoniae* in several countries (Loucif et al., 2016) (Cubero et al., 2015). Overall, a high level of genetic diversity was observed between the environmental Kp\_BM758 and the 34 Irish clinical *K. pneumoniae* and ST101 was not identified among this clinical *K. pneumoniae* collection. Correspondence with the NCPERLS has confirmed that to date two OXA-48-like-producing ST101 *K. pneumoniae* (both in 2019) have been identified in the Irish healthcare setting (unpublished data, NCPERLS). This indicates that this sequence type is present among clinical isolates of OXA-48-like-producing *K. pneumoniae* in Ireland. Kp\_BM758 harboured *bla*<sub>OXA-48</sub> on an IncL(pOXA-48) plasmid, the plasmid most commonly associated with carrying this gene, globally (HPSC, 2019) (Skalova et al., 2017) (Poirel et al., 2012). Analysis indicated that the IncL(pOXA-48) plasmid carried by Kp\_BM758 was not closely related to plasmids found within the clinical collection.

In conclusion, we report the detection of both OXA-48-like-producing ST131 *E. coli* (Ec\_BM707) and OXA-48-like-producing ST101 *K. pneumoniae* (Kp\_BM758) in Irish recreational water. Similarities were identified between the two environmental isolates and a collection of clinical CPE, with both collections harbouring *bla*<sub>OXA-48</sub> on similar mobile genetic elements. While Kp\_BM758 was genetically distant when compared to the 34 clinical *K.*

*pneumoniae*, a high level of genetic similarity was found between Ec\_BM707 and a number of clinical *E. coli*, with the nearest isolate matching at 98.2% of cgMLST loci. The findings of this study are a cause for concern, especially given the potential for the aquatic environment to act as a favourable setting for the horizontal gene transfer of these important mobile genetic elements between different bacterial strains and species. These findings highlight the need for the environment to be examined more closely for its role in the dissemination of AMR of public health significance.

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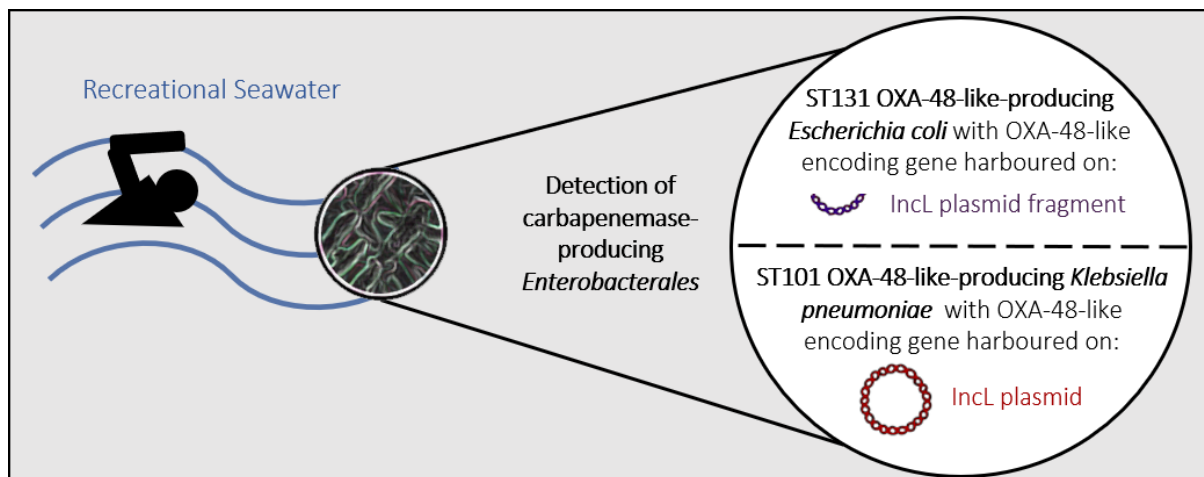
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**Table 1: Overview of results of antimicrobial susceptibility testing and *in silico* analyses of genomic data of OXA-48-like-producing *Enterobacterales* isolated from recreational water, Ireland, 2017.**

Isolate ID (sampling date)	Antimicrobial susceptibility testing	Sequence type	Plasmid replicons
Ec_BM707 (29/05/2017)	<b>Non- susceptible to:</b> ampicillin, cefpodoxime, cefotaxime, ceftazidime, ciprofloxacin, ertapenem, meropenem, nalidixic acid	ST131	Col156, IncFIA, IncFIB, IncFII
Kp_BM758 (14/08/2017)	<b>Non- susceptible to:</b> ampicillin, cefpodoxime, cefotaxime, cefoxitin, ceftazidime, chloramphenicol, ciprofloxacin, ertapenem, gentamicin, kanamycin, meropenem, nalidixic acid, tetracycline, trimethoprim	ST101	Col440II, IncFIA, IncFIB, IncFII, IncL(pOXA-48), IncR



Graphical abstract

**Highlights:**

- The aquatic environment can act as a reservoir for antimicrobial resistance
- Seawater samples were examined for carbapenemase-producing *Enterobacterales* (CPE)
- OXA-48-like-producing ST131 *E. coli* was detected in May 2017 (Ec\_BM707)
- OXA-48-like-producing ST101 *K. pneumoniae* was detected in August 2017 (Kp\_BM758)
- Similarities were identified between Ec\_BM707 and a collection of clinical CPE

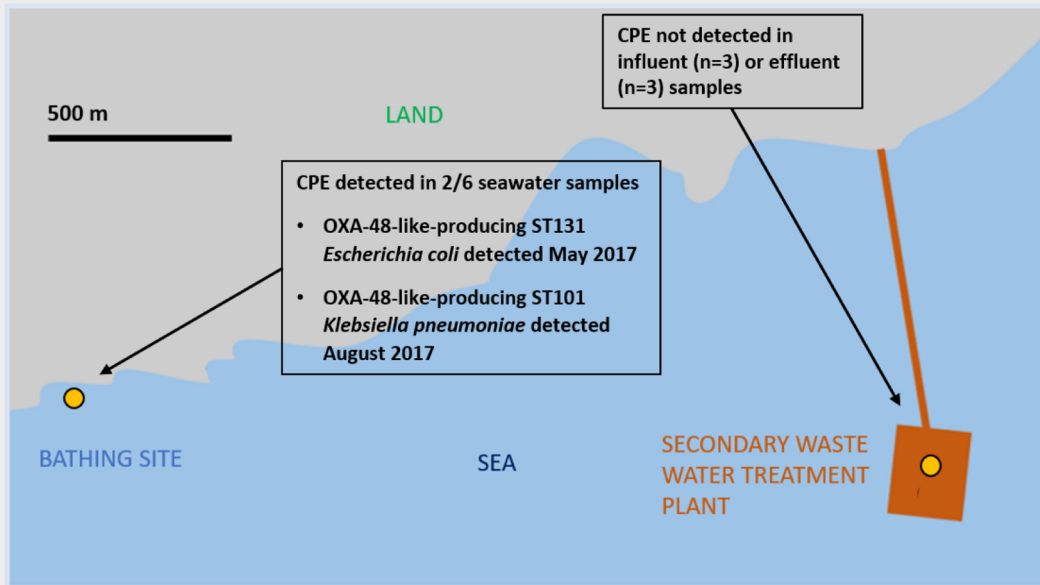


Figure 1

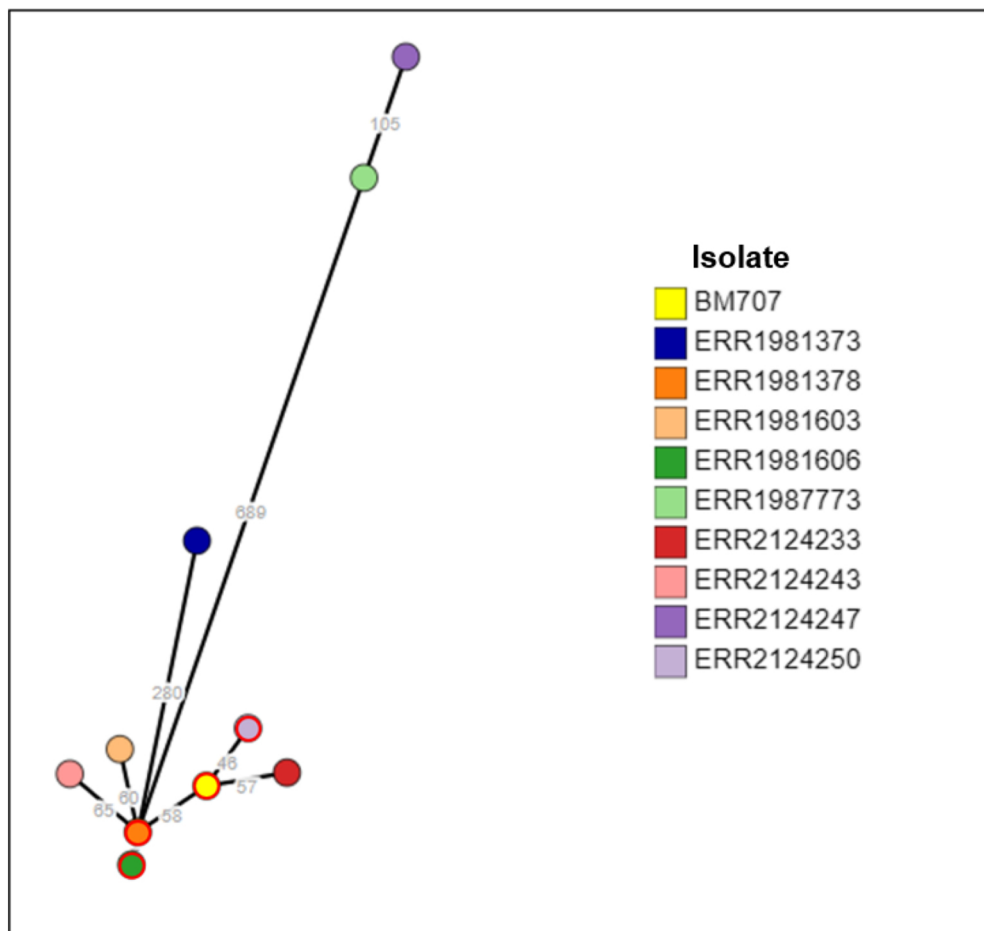


Figure 2