Dare to dive in? Antibiotic resistant bacteria in recreational water in Gothenburg, Sweden

Degree Project in 1 year Master programme in medical microbiology, with specialization in infection prevention and control, 15 hp

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ABSTRACT

BACKGROUND

Antibiotic resistant bacteria constitute a major global public health threat. Certain environments and settings contribute to the dissemination of such bacteria, and of antibiotic resistance genes. Understanding how pathogens acquire resistance, and when and where humans are exposed to such pathogens in a way that can cause colonization and infection, is crucial in tackling antibiotic resistance.

AIM

This study examined bacteria exhibiting resistance to fluoroquinolones or with phenotypic resistance resembling ESBL/ESBL$_{CARBA}$ in public swimming pools and in freshwater lakes used for recreational activities in the Gothenburg area.

MATERIALS AND METHODS

Water samples were cultivated on media selecting for gram negative and gram positive bacteria as well as enterococci. Gram negative bacteria were tested for resistance towards cefotaxime, meropenem, temocillin, and ciprofloxacin.

RESULTS

In all but one lake, bacteria exhibiting resistance to at least one antibiotic was found. Resistance against cefotaxime was most commonly found. Gram negative bacteria was more prevalent after incubation in 30 ℃ compared to 37 ℃. No viable bacteria was found in pools.

DISCUSSION

Chlorination of public pools seems effective in killing bacteria. Using freshwater lakes for recreational activities may constitute a risk for acquiring antibiotic resistant bacteria.

IMPLICATIONS

More research is needed to understand the risk for colonization and symptomatic infection associated with using lakes and pools for recreational activities. There is also a need to evaluate protocols for cultivation of water from lakes and pools.

BACKGROUND

Antibiotic resistance is an acute and rapidly growing problem, affecting people, animals, and communities (1). It is widely viewed as one of the most urgent public health threats globally. To hinder the progression and reduce the impact of antibiotic resistance, knowledge of its origin, development, and dissemination between bacteria, between geographical regions, as well as between clinical and non-clinical settings is essential. Some areas in these fields are well explored, while others require more research.

Even though antibiotic resistance in pathogens may have emerged after the utilization of antibiotics in medicine and animal production began, antibiotic resistance genes (ARGs) themselves can be ancient (2). Phylogenetic and metagenomic studies indicate that some resistance genes, for example β-lactamases, originated and spread throughout environmental bacteria long before the discovery of antibiotics by humans (3-6). However, our current use of these drugs has likely contributed to the accelerated horizontal gene transfer (HGT) of ARGs between different bacterial species, including pathogens. HGT is a result of transduction, transformation or conjugation which ensues in an exchange of genetic material between bacteria (7). For HGT to occur, the donating and receiving bacteria must be in the same environment (8). Transfer is more likely between bacteria that are phylogenetically closely related, and when stressors are present in the environment (9, 10). It has been shown that stress by for example ciprofloxacin can lead to DNA damage and promote transfer of integrating conjugative elements which carry several ARGs (11).

ARGs can be located either on the bacterial chromosome or on mobile genetic elements (MGEs) (8). Typically, ARGs on MGEs will either be associated with a negligible fitness cost, or, if the fitness cost is not negligible, with a selection pressure contributing to keeping the gene in place. Certain environments, for example sewage treatment plants or farms, are more likely to present such a selection pressure through the presence of an adequate concentration of antibiotics, than other environments, such as glaciers or non-agricultural soils (3, 4). In a heavily antibiotic-polluted lake, both ARGs and MGEs were found in high abundance compared to in a non-polluted lake (12). Such environments present a higher risk for transfer of resistance to clinically relevant bacteria (4). Commonly, more than one ARG is found within the same area of the genome, such as the *Salmonella* genetic island 1 found among *Salmonella enterica* (13). These multi-drug resistance encoding areas are frequently associated with integrons (genetic elements allowing acquisition and expression of gene

cassettes) and transposons (a type of MGE). Selection for resistance against one type of antibiotic is thereby likely to select for resistance against other antibiotics, as the different ARGs are often inherited or transferred together. This leads to a more extensive enrichment of ARGs. Even in the absence of antibiotics, other environmental factors may indirectly result in maintenance of ARGs, as the MGEs they reside on often are associated with genes for heavy metal and biocide resistance (3, 4, 8). Other genes giving the host an advantage, such as genes for toxin production, may also be located on the same MGE (3). Antibiotic resistance can therefore be associated with increased virulence, and environmental pollution can maintain the presence and spread of resistance.

Antibiotic resistance can develop as a result of mutations and spread within a clone, and can also be acquired by HGT of ARGs between different bacterial species. It can arise through two main mechanisms: through reduction of the concentration of the drug at the target site (obtained through efflux pumps expelling the drug, a modified cell wall reducing the ability of the drug to penetrate and thereby reaching its target, or enzymes that inactivate the drug), or through altering the drug target (target mutation, target replacement, target protection, or enzymatic alteration of the target) (4). For example, β-lactam resistance is a result of either an altered penicillin-binding protein (PBP) inhibiting the interaction between penicillin and PBP, inaccessibility to PBP by increased activity of efflux pumps or decreased numbers of porins in the cell wall, or by drug inactivation through β-lactamases (13). As these and other mechanisms for resistance spread among (potential) pathogens, antibiotics used in clinical settings become increasingly ineffective.

The latest class of antibiotics with a previously clinically unused mechanism of action, oxazolidinones, was discovered in the 1980's. Explanations for the lack of development of new antibiotics include the extensive resources required to identify suitable drug candidates, less future profit for pharmaceutical industries (any new drugs would require prudent use to ensure their sustained efficiency, costly clinical trials, short treatments compared to other types of drugs, etc.). Norrby et al. suggest a higher level of collaboration between academia, pharmaceutical and biotechnological companies as well as improved study design of clinical trials and legislation encouraging and facilitating the discovery and development of new drugs (14).

The same ARGs can be found in soil bacteria as in pathogens (2, 15). Resistance genes that are identical when comparing nucleotides, and MGEs surrounding such areas in the genome in these different bacteria are proof of recent events of HGT (15). Non-clinical environments

can therefore be seen as a reservoir for known and unknown ARGs (3, 13, 16). Exposure of these environments to antibiotics or other stressors would therefore increase the risk of dissemination of resistance within and between bacterial communities. The levels of ARGs in soil have slowly increased over time since the beginning of the antibiotic era (17). Antibiotics may enter the environment through many different routes, for example from manure and urine originating from production animals administered antibiotics (18). Application of manure to agricultural land, farm runoffs and irrigation with wastewater can contaminate environments such as soils and waters. It has been estimated that up to 75% of antibiotics distributed orally to feedlot cattle may be excreted in active form (19). In 2012, approximately 32.2 million pounds of active antimicrobial ingredients for use in animals were sold in the U.S., to be compared to 7.25 million pounds for use in humans.

The use of certain antibiotics as growth promoters in the animal production industry has been shown to increase resistance to other antibiotics in human pathogens (20). For example, avoparcin is such an antibiotic, which selects for vancomycin resistant *Enterococcus* (VRE). Where avoparcin has been used, carriage of VRE is much more common both in humans and in production animals and pets than in, e.g., Sweden and the US where avoparcin has not been used. The usage of avoparcin on individual farms is correlated to a significantly higher prevalence of VRE in the gut flora of the animals on such farms, compared to farms where avoparcin is not used (21). Antibiotics that are used for therapeutic care in humans, also those that are described by the U.S. Food and Drug Administration as highly important and even critical, are today used for growth promotion in the United States (18). β-lactam resistance, such as extended spectrum β-lactamases (ESBL), has been increasingly reported from production animals (13). Pathogens of animal origin and with zoonotic potential include *Escherichia coli*, *Salmonella*, *Campylobacter*, *Staphylococcus aureus*, *Enterococcus*, *Haemophilus influenzae*, *Mannheimia hemolytica* and *Pasteurella multocida*,

Humans encounter resistant bacteria mostly through contact with other humans (8). Transmission can occur in clinical as well as non-clinical settings. Antibiotic resistant bacteria can also spread from animals to humans in different ways, such as through contaminated food of animal origin (18, 22). For example, an outbreak of plasmid-mediated multi-resistant *Salmonella typhimurium* in a Canadian nursery ward was shown to have started on a local dairy farm (23). The mother of the index patient had consumed unpasteurized milk from the farm and was asymptomatically shedding the bacteria upon delivering her baby who then became infected. Through suspected cross contamination, *S. typhimurium* infected other

patients in the ward. In 1998, a Danish outbreak of multidrug-resistant and quinolone-resistant *S. typhimurium* was proven to have spread from two pig herds, through the food chain to several persons (24). An American study found an association between methicillin resistant *Staphylococcus aureus* (MRSA)-infections in humans and proximity to high density livestock production, particularly pig production and the application of manure in crop fields from such facilities (21). MRSA has been shown to be transmissible from pigs to pig farmers, and then within and beyond the family of the farmers (25). This confirms that transmission between animals and humans can also occur through both direct and indirect contact. Farmers, people visiting or working on farms, or who share household with such persons are more likely to acquire an antibiotic resistant bacteria derived from production animals, than is the population in general. This can pose a major threat to the health of individuals, as infections become challenging to treat. For example, *Salmonella* and *Campylobacter* are common pathogens associated with foodborne illness in children (18). In 2013, it was estimated that 8% respectively 24% were drug resistant. Alarmingly, 2-3% of tested isolates were resistant to the preferred antibiotic treatment in pediatric infections.

Another route of exposure to resistant bacteria is through water. Shuia *et al.* showed that swimming in public swimming pools alters the skin microbiome and the composition of ARGs present on the skin (26). Swimming pools may be a potential health risk for acquiring ARGs. However, the study provided no evidence of viable bacteria as the authors did not perform any cultivation. Recreational activities involving coastal ocean water have been assumed to provide a route of exposure to *E. coli* exhibiting resistance to third generation cephalosporins, through ingestion of water (27). Participation in these types of activities has also been associated with an increased risk of illness (symptoms from ears, gut, and any illness) when compared to members of the public not exposed to ocean water (28). However, such differences could be caused by lifestyle factors, with exposure to ocean water simply being a confounder.

Freshwater bodies, particularly rivers and lakes, can exhibit high amounts of antibiotic resistant bacteria compared to other natural environments, thereby having the potential to function as reservoirs for ARGs (29, 30). ESBL- and $ESBL_{CARBA}$-producing Enterobacteriaceae, as well as MRSA and VRE, are examples of resistant bacteria that have been found in freshwater across the globe and that pose a great threat to healthcare (29). The presence of antibiotics in the environment contributes to the selection of ARGs, depending on the concentrations of these selective agents. A wide variety of different antibiotics have been

detected both in water and sediments as well as in aquatic plants, algae, and animals from different lakes around the world (31). Antibiotics can reach freshwater bodies from farmland run-off, wastewater treatment plants, sewage water, effluent water from hospitals, drug factories, animal and human feces, but also from antibiotic-producing environmental microbes (29, 30). These environments can allow for HGT to pathogens to occur, as a selection pressure in the form of antibiotics, bacteria carrying ARGs, as well as pathogenic bacteria potentially receiving ARGs are all present in the same settings. (29). Humans may be exposed to antibiotic resistant bacteria and ARGs when swimming in freshwater, when consuming freshwater fish and when freshwater is used for irrigation or as drinking water.

Determining the background levels of antibiotic resistance in non-clinical environments, for example lakes, where humans (and animals) are at risk of acquiring resistant bacteria, which can be pathogenic, commensal or opportunistic, will help in designing interventions to avoid transmission of antibiotic resistance in non-clinical settings (3). However, most research in the field of antibiotic resistance has been focused on the clinical environment. To safeguard our antibiotics, thereby ensuring the availability of efficient treatments for infections now and in the future, we must understand how antibiotic resistance emerges and what factors contribute to the dissemination of ARGs. This study aims to contribute to increased knowledge in this area by addressing antibiotic resistant bacteria present in public pools and in freshwater lakes where recreational water activities take place.

RESEARCH QUESTION

The aim of this study is to determine the presence or absence of viable bacteria exhibiting resistance to fluoroquinolones or producing ESBL/ESBL$_{CARBA}$ in public swimming pools and in freshwater lakes used for recreational activities in the Gothenburg area.

MATERIALS AND METHODS

Sampling and analysis were carried out in accordance with the protocols employed in the EMBARK project (http://antimicrobialresistance.eu). This thesis is part of the work performed in EMBARK.

In and around Gothenburg, 23 public pool facilities were invited to participate in our study. Twelve facilities accepted. Depending on the type and number of pools at each facility, one or more pools of varying temperature were sampled. Sampling of each pool was done in the morning when none or few visitors had entered the pool, and again in the afternoon after the pool had been in use during the day. In addition, tap water was also sampled once at every facility as a control. From each pool, a 50 ml Falcon tube of surface water was collected. Falcon tubes were filled with lukewarm water from taps.

Thirteen freshwater lakes in and around Gothenburg commonly used for swimming were also sampled. For each lake, a 50 ml Falcon tube was filled with surface water from the shoreline at each location. Sampling was carried out in February and March of 2022. After the initial sampling, a follow-up sampling was conducted at three of the initially sampled lakes to allow comparison of results from this study with results from water quality control conducted by municipalities. Again, surface water from the shoreline was collected, and also water from around 30 cm depth where the lake was around 1 m deep. The latter samples were collected from docks adjacent to the swimming areas. When the municipalities sample water, they collect samples from 30 cm depth where the water is 1 m deep(32). The follow-up sampling was carried out in April 2022. All samples were transported directly to the laboratory for plating or stored overnight at 4 ℃ before being plated at the laboratory.

For each sample, a set of 6 or 7 different media were used (Table 1). Media was provided by Sahlgrenska University Hospital, Clinical Microbiology, Substrate Service. The antibiotics used for culturing gram negative (G-) bacteria have been chosen with relevance to public health and infection control in mind (Personal communication E Ruppé 28 Apr 2022). G-bacteria producing ESBL or $ESBL_{CARBA}$ are currently regarded the largest problem globally when it comes to antibiotic resistance. According to guidelines published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), meropenem is the antibiotic of choice for detecting carbapenemase production, and resistance towards cefotaxime indicates (but does not confirm) production of ESBL (33). Temocillin is used to screen for the carbapenemases OXA-48 and OXA-181, which are usually sensitive to meropenem (Personal communication E Ruppé 28 Apr 2022). Ciprofloxacin indicates resistance towards fluoroquinolones, which is one of the most commonly used classes of antibiotics worldwide, justifying its inclusion in this protocol. The concentrations of antibiotics in the different media, as seen in table 1, are chosen to comply with World Health Organization's Global Antimicrobial Resistance and Use Surveillance System. Not all samples were cultured on Bile

Esculin Agar (BEA) due to problems with agar plate delivery. For pool facilities, see Table 2. No lake samples from the initial sampling were cultured on BEA. One lake sample (St Stamsjön) was not cultured on MacConkey with meropenem, cefotaxime nor ciprofloxacin at 37 °C due to media accidentally being mixed up during plating.

Table 1. Media used for culturing.

Type of media	Abbreviation*	Type of bacteria selected for
MacConkey	MC	G-
MacConkey with 32 mg/L temocillin	MC+t	G- with resistance to carbapenem
MacConkey with 0.25 mg/L meropenem	MC+m	G- with resistance to carbapenem
MacConkey with 1 mg/L cefotaxime	MC+ct	G- with resistance resembling ESBL
MacConkey with 0.125 mg/L ciprofloxacin	MC+cp	G- with resistance to fluoroquinolone
Bile Esculin Agar	BEA	Enterococci
Mannitol Salt	MS	G+

*Abbreviations are used in Table 3 and in Figure 1. G- = gram negative, G+ = gram positive.

Falcon tubes containing follow-up samples from lakes were vortexed before cultivation. 100 µL of each water sample from pools and lakes was spread on two plates of each media. Plates were incubated for 24 h at 30 ℃ and 37 ℃, respectively. Temperatures were chosen with respect to previous work in EMBARK, which has found 30 ℃ and 37 ℃ to yield the highest abundance of bacterial growth (Personal communication J Bengtsson-Palme 8 Feb 2022). After incubation, the colony forming units/mL (cfu/mL) on each plate were counted.

As we were collecting and analyzing data from pool facilities, we noticed no growth on any of our plates. Therefore, the protocol for cultivation was slightly altered to maximize chances of finding viable bacteria (Table 2). For facilities A-F, cultivation was performed as described above. For facilities G and H, 1 ml av water from each sample was plated instead of 100 µL. Plates were dried in fume cupboard during 2 hours before incubation. For facilities I-L, sodium thiosulphate at a concentration of 100 mg/L was added upon arrival to the lab. Sodium thiosulphate inactivates chlorine, which is commonly used as a disinfectant in public pools.

Table 2. Sampled pool facilities.

Pool facility	Sampling sites	Temperature (°C)	Comments
A	Exercise pool, tap water	26, lukewarm	
B	Exercise pool, teaching pool + children's pool, tap water	28, 34.5, lukewarm	
C	Adventure pool, jacuzzi, tap water	27, 37, lukewarm	
D	Exercise pool, tap water	28, lukewarm	
E	Adventure pool, teaching pool, jacuzzi, tap water	28, 34, 37 lukewarm	
F	Exercise pool, tap water	27.5, lukewarm	Facility uses salt water instead of fresh water in pool
G	Teaching pool, tap water	34, lukewarm	Not cultivated on BEA; 1 ml of sample/plate
H	Exercise pool, teaching + children's pool, tap water	26.5, lukewarm	Not cultivated on BEA; 1 ml of sample/plate
I	Exercise pool, tap water	28, lukewarm	Sodium thiosulphate was added
J	Teaching pool, tap water	34, lukewarm	Sodium thiosulphate was added; only morning samples were taken as the pool had not been cleaned following use the previous day
K	Exercise pool, one cooler and one warmer teaching pool, tap water	27, 29, 32, lukewarm	Sodium thiosulphate was added
L	Exercise pool, tap water	27, lukewarm	Sodium thiosulphate was added

The temperatures are presented in the same order as the corresponding sampling sites for each facility.

Comments indicate when a change of protocol was carried out.

ETHICS

Ethical approval is not needed for water samples. Data from pool facilities has been de-identified so that outcome will not risk causing financial or other type of harm to businesses or individuals.

RESULTS

Among the samples collected from pool facilities, only two were found to contain cultivable bacteria. The sample from the 34 ℃ pool at facility N had one colony, equivalent to ~10 cfu/mL, on the mannitol salt agar after being incubated for 72 h at 30 ℃. The initial reading after 24 h incubation was uninterpretable (it was unclear if colonies had grown or not), and therefore the plate was incubated over the weekend. The tap water sample at facility L showed growth at 37 ℃ on MacConkey as well as MacConkey + meropenem. Both plates exhibited a "bacterial lawn" (BL), meaning colonies were indistinguishable from each other and could not be counted.

In the initial sampling of lake water, bacteria were found in all samples but one (Table 3). Growth on MacConkey agar selecting G- bacteria ranged from 0 to 1300 cfu/mL (2 samples being negative) at 30 ℃, and from 0 to 670 cfu/mL (10 samples being negative) at 37 ℃. On Mannitol salt selecting for G+ bacteria, 0-20 cfu/mL (10 samples being negative and 1 uninterpretable) was seen at 30 ℃, and at 37 ℃ seven samples were negative, three had 10 cfu/mL, and three exhibited BL. Growth on MacConkey + temocillin was seen in 8 and 1 samples incubated at 30 and 37 ℃, respectively. One sample incubated at 30 ℃ was uninterpretable. Growth on MacConkey + meropenem was seen in 10 and 0 samples incubated at 30 and 37 ℃, respectively. One sample incubated at 37 ℃ was uninterpretable. Growth on MacConkey + cefotaxim was seen in 11 and 2 samples incubated at 30 and 37 ℃, respectively. One sample incubated at 37 ℃ was uninterpretable. Growth on MacConkey + ciprofloxacin was seen in 2 and 1 samples incubated at 30 and 37 ℃, respectively. One sample incubated at 30 ℃ was uninterpretable. Samples were uninterpretable due to accidentally being thrown out before being read.

Table 3. Bacterial growth by media and temperature for each lake.

The first table shows the initial sampling of all lakes, the second table shows follow up sampling including sampling site for three lakes. Growth is displayed in cfu/mL for each sampled lake by media and temperature. See Table 1 for types of media. Temperature in °C. X = sample not cultivated due to technical difficulties or uninterpretable. Orange cells indicate growth, green cells indicate no growth.

	Media/temperature											
Lake	MC 30	MC 37	MC+t 30	MC+t 37	MC+m 30	MC+m 37	MC+ct 30	MC+ct 37	MC+cp 30	MC+cp 37	MS 30	MS 37
Aspen	730	670	460	0	100	0	250	0	70	60	20	0
St Mölnesjön	90	10	30	0	520	0	180	0	0	0	0	10
Surtesjön	10	0	0	0	10	0	10	0	0	0	0	0
Kåsjön	0	0	0	0	0	0	0	0	0	0	0	0
St Stamsjön	300	30	100	10	830	X	410	X	0	X	0	BL
Bergsjön	490	0	0	0	30	0	0	0	0	0	0	0
Härlanda tjärn	1080	0	70	0	50	0	70	0	0	0	0	0
Delsjön	0	0	0	0	0	0	20	0	0	0	10	BL
Sö Långvattnet	50	0	1140	0	200	0	1540	0	0	0	0	0
Stensjön	630	0	1200	0	0	0	2300	0	0	0	0	BL
Rådasjön	350	0	270	0	60	0	180	0	0	0	X	10
Finnsjön	1300	0	X	0	10	0	100	10	X	0	0	0
Tulebosjön	470	0	1920	0	1120	0	1460	30	20	0	0	10
Prevalence	0,846	0,23	0,67	0,1	0,77	0	0,85	0,17	0,17	0,08	0,17	0,46

	Media/temperature													
Lake	MC 30	MC 37	MC+t 30	MC+t 37	MC+m 30	MC+m 37	MC+ct 30	MC+ct 37	MC+cp 30	MC+cp 37	BEA 30	BEA 37	MS 30	MS 37
Kåsjön dock	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kåsjön shore	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Härlanda dock	10	0	0	0	0	0	10	0	0	0	0	0	0	0
Härlanda shore	40	20	0	0	10	0	0	0	0	0	0	20	0	10
Delsjön dock	20	0	0	0	0	0	10	0	0	0	0	0	0	0
Delsjön shore	730	120	670	0	280	10	570	20	0	0	0	0	0	20

Table 3 also shows the results from the follow up sampling. Again, Kåsjön, which did not display any bacterial growth in the initial sampling, was without growth. Härlanda displayed a marginally higher growth at the shore compared to the dock, whereas Delsjön showed a considerably higher bacterial concentration at the shore. In all lake samples combined, G- bacteria grew in larger numbers when incubated at 30 °C, whereas G+ bacteria were more prevalent when incubated at 37 °C.

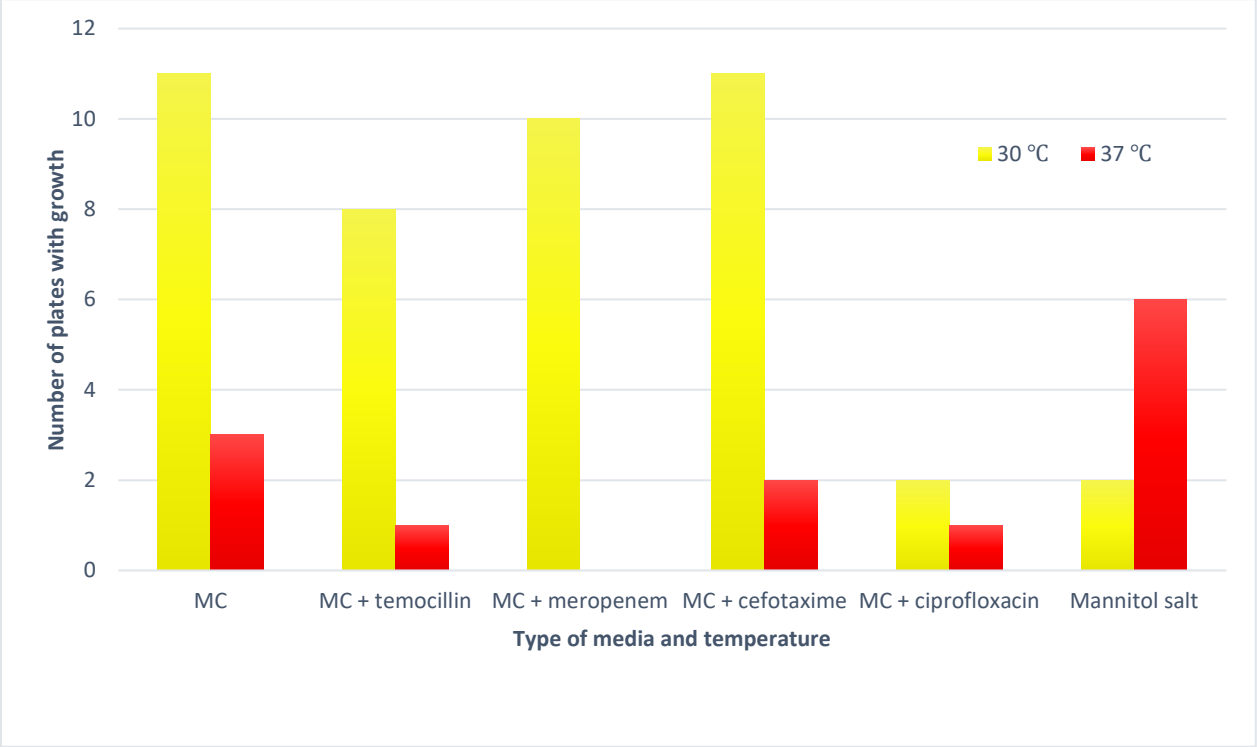

Figure 1. Bacterial growth by media and temperature.

Number of plates incubated with initial lake samples displaying bacterial growth. Note that not all samples were incubated on all types of media due to technical difficulties, and not all plates were interpretable.

Table 4. Prevalence of plates from the initial lake samples displaying bacterial growth by media and temperature

Type of media	30 °C	37 °C
MacConkey (MC)	85%	23%
MC + temocillin	67%	8%
MC + meropenem	77%	0%
MC + cefotaxime	85%	17%
MC + ciprofloxacin	17%	8%
Mannitol salt	17%	46%

The most common antibiotic resistance in lakes among bacteria incubated at 30 ℃ was towards cefotaxime (85%), followed by meropenem (77%), temocillin (67%) and the least common out of the four antibiotics used was ciprofloxacin (17%). Prevalence has been calculated using results from the initial sampling. For bacteria incubated at 37 ℃, the pattern was the same, although the frequency was lower and resistance towards meropenem was not detected.

In summary, antibiotic resistant G- bacteria were found in all but one lake, and in one of the pool facilities (in tap water) but in none of the sampled pools. Resistance in lake samples was found towards all the types of antibiotics tested, and for temocillin, cefotaxime and ciprofloxacin growth was observed both at 30 and 37 ℃. In one tap water sample, G- bacteria grew at 37 ℃, both on plates without any antibiotic as well as with meropenem.

DISCUSSION

This study found that freshwater lakes used for recreational activity in the Gothenburg area harbour much more cultivable bacteria, including bacteria resistant to cefotaxime, meropenem, temocillin and ciprofloxacin, than public swimming pools in the same area. It seems that chlorination is very effective in killing such bacteria, as we observed no differences in pool samples taken before and after visitors had used the pools.

In the sampled lakes, G- bacteria were found in larger numbers and with higher prevalence when incubation was performed at 30 °C compared to 37 ℃. The opposite was found for G+ bacteria. It is reasonable to assume that bacteria growing at 30 ℃ would be more likely to be of types that thrive in the environment but may potentially not pose the same direct threat to human health as bacteria growing at 37 ℃, which are more likely to be human pathogens. However, many bacteria are able to grow at 30 °C even if their optimal growth temperature is at 37 °C. In addition, the antibiotic resistant bacteria growing at 30 ℃ may indirectly contribute to antibiotic resistance in human-associated bacteria. Previously, it has been shown that lakes containing large amounts of ARGs also contain MGEs (12). This would allow for events of HGT to human-associated bacteria when environmental bacteria come in contact with the human microbiome. Such contact could occur either on the skin or in the gut through ingestion of water. Recreational activities involving ocean water may be associated with illness of the ears and gut (28). One study identified a higher risk for gut colonization with *E. coli* carrying ESBL among surfers compared to non-surfers in England and Wales (34).

Swimming in the ocean has also been shown to alter the skin microbiome and increase the abundance of ARGs and genes for virulence factors found on the skin (35). ARGs were acquired from the ocean water, and multidrug resistance genes increased by more than 40%. Ocean bacteria were still found on the skin 24 hours post exposure, which makes HGT possible. Swimming in the ocean can provide a risk of acquiring antibiotic resistant pathogens on the skin, which in turn may potentially lead to skin infections. Similar studies on lake water have to date not been carried out as far as I am aware of. However, lakes used for recreational activities are known to contain pathogenic bacteria as well as ARGs (36, 37). Assuming that lakes in this study are representative for freshwater swimming bodies in the Gothenburg area, potential pathogens with antibiotic resistance to temocillin, cefotaxime and ciprofloxacin can be expected to be commonly encountered, and recreational use of lakes in this region may increase the risk of acquiring antibiotic resistant infections. However, before the level of risk for such events can be determined, more studies addressing, e.g., the pathogenicity of ocean and lake bacteria, the survival of such bacteria on the skin, the infectious doses required for colonization and symptomatic infection, the pathogenicity of bacteria of the human microbiome recipients of HGT of ARGs, etc., are required. Similar studies are also needed to evaluate the risk of symptomatic gut infections through ingestion of water, or risks of other illnesses that can be associated with the recreational use of water in lakes or oceans. It is important to note that this study only evaluates phenotypic resistance to antibiotics, and we cannot draw any conclusions regarding types of or abundance of ARGs, as the resistance found through cultivation could also be caused by mutations or intrinsic resistance.

The most common type of resistance detected in the lakes included in this study was against cefotaxime. Resistance against meropenem and temocillin was also quite common, whereas resistance towards ciprofloxacin was less common. Even though the prevalence of cefotaxime resistant bacteria was high among the sampled lakes, it may not have been the most abundant type of resistance among bacteria within each lake. Several lakes are likely to have contained larger quantities of bacteria with other phenotypic resistances than against cefotaxime. Furthermore, this study does not address all types of antibiotic resistance. For example, we did not use any media containing tetracyclines or sulfonamides. In a recent study of a Chinese lake, ARGs against these antibiotics were the most abundant (38). In that study, the temperature, amount of antibiotics in the lake and the abundance of ARGs and certain MGEs increasing the risk for events of HGT, varied with season. A positive correlation between

most studied ARGs and antibiotics, water temperature and nutrients (the studied lake being eutrophic), as well as a negative correlation to dissolved oxygen levels, was seen. This implies that the risk of acquiring ARGs when using a lake for recreational purposes also varies depending on the time of year. Our study was unfortunately not carried out during the summer, when swimming outdoors is most common, and the distribution of resistant bacteria might be different during this period.

During the summer, Swedish municipalities frequently analyze water samples from popular swimming areas to evaluate bathing water quality. The results from cultivation of lake samples from this study can be compared to the official results for the summer of 2021, available through the Swedish Agency for Marine and Water Management. For results from lakes that were also represented in this study, see Appendix 1 (note that the unit used for *E. coli* and intestinal enterococci is cfu/100 ml). This corresponds to levels of detected *E. coli* ranging from <0.01 to >10 cfu/ml, mostly in the range around 0.1 cfu/ml, whereas our study found G- bacteria in 0 to >1000 cfu/ml, mostly in the range of hundreds of cfu/ml. Since no further identification of bacterial species was conducted in this study, we do not know how many of the bacteria growing on the MacConkey agar were *E. coli* specifically. It can nevertheless be concluded that this study found significantly larger numbers of bacteria through our sampling methods compared to official sampling results. As described by the Swedish Agency for Marine and Water Management, sampling by the municipalities is to be performed at a location where water depth is at least 1 m, and the sample should be taken from 30 cm below the surface (32). This might explain the difference in results between this study and official results, as this study mostly sampled water from the surface adjacent to the shoreline of each lake. Indeed, the follow up sampling in this study indicates that there might be more bacteria found at the shoreline than further out and deeper in the water. However, as only three lakes were sampled with the purpose of evaluating this aspect, a larger study would need to be carried out before any certain conclusions can be drawn. It can be argued that the method of sampling at the shoreline in this study is more relevant to a specific category of people using lakes for recreation, namely babies and young children who most often will play in shallow waters along the shoreline. This group of children might also be more likely to swallow water than older children and adults, suggesting a possible higher exposure of antibiotic resistant bacteria.

Among 18 samples of pool water from 12 different pool facilities, only one sample contained low levels of G+ bacteria (~10 cfu/mL). Due to difficulties in reading the plate after 24 h, it

was incubated over the weekend for a total of 72 h. All other plates were incubated for 24 h, and it is uncertain whether bacteria would have grown also on other plates if they had been allowed to grow for longer. No viable G- bacteria were found in any of the pool samples, and therefore no antibiotic resistance was detected. After cultivating samples from 6 facilities and not detecting any bacteria, we altered the laboratory protocol in two different ways (Table 2) to improve the detection limit of our methods. Despite increasing the sample volume cultivated for 4 of the remaining samples, and adding sodium thiosulphate to the last 4 pool samples, no difference was seen on the outcome. Due to small sample numbers and growth only in a single sample, no certain conclusions regarding the ideal cultivation method can be drawn from this study.

A Chinese study of microorganisms in public outdoor swimming pools found viable coliforms and *Pseudomonas aeruginosa* in 10.3% and 69.2 % of samples respectively (39). Significantly larger volumes of pool water were used in that study (50 L), and samples were collected from the bottom of the pools. Sodium thiosulphate was added to each sample. The samples were filtered and the retentate was suspended in broth and incubated for 24-48 h at 37 ℃ before being cultivated on other media than the ones used in this study. Total coliform bacteria were found at a concentration of up to 154 most probable number (MPN)/100 mL, which corresponds to 0.15 bacteria/100 µL (100 µL being the volume used for cultivation in this study). If the levels of coliform bacteria in this study are comparable to the highest concentrations found in the Chinese study, we would have expected to find around 2-4 cfu in total in the 19 pools sampled. As we found no coliform bacteria (no growth on MacConkey media), there seems to be less bacteria in the Swedish pools studied than in the Chinese pools. However, these results may be coincidental, given the uncertainty associated with small numbers of observations. More sampled Swedish pools would be needed to properly perform a comparison between the studies. A Portuguese study used 1000 mL of indoor pool water collected 10-20 cm below the surface without adding sodium thiosulphate (40). Microbial analysis was carried out within 2-4 days of sampling. 100 mL of each sample were filtered, and the filter membrane was placed on different media selective for *E. coli* and *P. aeruginosa* before incubation at 37 ℃ for 24 h. Out of 60 pool samples, one contained *Vibrio* spp. A Turkish study used yet another method, where 8 L of water from 1 m below the surface was collected from each of 13 different public indoor swimming pools (41). Samples were filtered and the filter papers were incubated in brain heart infusion broth for 24-48 h before 100 µL of the suspension was cultured on different media at 35 ± 2 ℃ for 1 week. Bacterial growth was

evaluated daily. In 53.8 % of samples, *Streptococcus viridans, Staphylococcus epidermidis, Pseudomonas stutzeri, Cryptosporidium* and/or *Bacillus* spp. were found.

The above-mentioned studies show a variety of different methods for detecting viable bacteria in pool water. Unsurprisingly, the results in the studies vary significantly. Using a different approach, for example collecting larger volumes of water and filtering before microbial analysis, or incubating samples in broth before cultivation, might have allowed detection of bacteria also in this study. Furthermore, even if no viable bacteria are detected using cultivation methods, there might be extracellular DNA present in the swimming pools, allowing for events of HGT. It has been shown that water chlorinated at levels sufficient to inactive VRE can still contain the ARG *vanA* (42). Chlorination of drinking water has also been shown to increase the total abundance of ARGs (43, 44). One study found that the resistome was profoundly altered, with genes encoding multidrug resistance increasing, and resistance genes towards tetracycline and sulfonamide decreasing (43). Also, the abundance of *Pseudomonas* and *Acidovorax*, bacteria that are known to carry many ARGs, increased after chlorination.

A large variability in cfu counts within samples was noted among the lake samples in this study. Our approach to analyzing samples does not with certainty tell us the abundance of resistant bacteria, and results might more appropriately be interpreted as presence or absence of (resistant) bacteria. For some lakes, particularly Stensjön and Södra Långvattnet, bacterial growth was seen in larger numbers on MacConkey media containing antibiotics than on antibiotic-free MacConkey plates. An explanation for this could be that the water samples were not homogenous upon being spread on the agar plates. Indeed, despite the use of sterile glass beads to spread samples as evenly as possible, many plates exhibited colonies concentrated to one area and within proximity of each other. This could be caused by biofilm keeping bacteria together. Therefore, the (lack of) presence of cultivable bacteria on each plate may be more informative than the specific bacterial counts. This might also be a source of error where bacteria were not growing on selective plates, as there might have been relevant bacteria within the samples that were not successfully transferred onto the plates.

Antimicrobial resistance, including antibiotic resistance, is by many viewed as a silent pandemic with potentially catastrophic consequences. For the individual patient, it increases the risk of morbidity and mortality (18, 45). With an increased burden of disease in the communities, poverty will grow disproportionately in low-income areas. Socioeconomically vulnerable individuals and groups are most likely to be affected by poverty caused by

antimicrobial resistance (46). Healthcare systems are expected to become destabilized as hospital stays become longer and more costly when treating infections not susceptible to standard antibiotic treatment protocols (1). The World Bank estimates that if left without meaningful action, antimicrobial resistance will by 2050 have pushed 28 million people into poverty, increased healthcare costs globally with up to 1 trillion USD, and will cause a decline in livestock production of up to 7.5% (47). Efforts must be focused on understanding and managing antimicrobial resistance today and in the future. This study highlights the importance of surface water in the spread of antibiotic resistant bacteria. Given that the majority of the Swedish lakes, which are often being monitored for water quality and are generally not directly used as drinking water, contained cultivable resistant bacteria, this problem might be substantially greater in low-income countries. It is therefore important to develop monitoring solutions for antibiotic resistance which are cheap and easily implementable also in low-resource settings, to allow science-driven policy and decision making to protect public health.

CONCLUSIONS AND IMPLICATIONS

In lakes used for recreational activity in the Gothenburg region, bacteria with phenotypic resistance against cefotaxime, meropenem, temocillin and ciprofloxacin were found. Exposure to water from these lakes may constitute a risk of acquiring antibiotic resistant bacteria. To evaluate the risk of morbidity following such exposure, more research is needed.

There is a need to evaluate cultivation protocols both for pools and surface water. Using our protocol, virtually no bacteria was found in pool water. There might have been viable bacteria in the pool water samples that we were unable to detect, or ARGs in the form of extracellular DNA. At the same time, our method detected higher abundances of bacteria in lake water than the standard methods used by municipalities in monitoring. This highlights the need for scientific evaluation of protocols for monitoring of antibiotic resistance in water.

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APPENDICES

Appendix 1. Data from lakes sampled by municipalities 2021, available through the Swedish Agency for Marine and Water Management.

Län	Kommun	Badplats	Datum	Eu-bad	*E. coli* prefix	*E. coli*	Intestinala enterokocker prefix	Intestinala enterokocker	Vädertyp	Kvalitet	Algblomning	Vattentemperatur	Latitud	Longitud	Badplatstyp
Västra Götalands	Göteborg	Stora Mölnesjön	2021-08-20	N	=	198	=	130	Viss molnighet	Tjänligt m. Anm.	Ingen blomning	17,0	57,805124000	12,075005000	Sjö
			2021-08-13	N	=	120	=	30	Mulet	Tjänligt m. Anm.	Ingen uppgift	19,5	57,805124000	12,075005000	Sjö
			2021-08-10	N	=	328	=	260	Mulet	Tjänligt m. Anm.	Ingen uppgift	19,0	57,805124000	12,075005000	Sjö
			2021-07-30	N	=	272	=	250	Viss molnighet	Tjänligt m. Anm.	Ingen blomning	20,6	57,805124000	12,075005000	Sjö
			2021-07-28	N	>	1 000	>	1 000	Mulet	Otjänligt	Ingen blomning	21,8	57,805124000	12,075005000	Sjö
			2021-07-23	N	=	540	=	70	Ingen uppgift	Tjänligt m. Anm.	Ingen uppgift	0,0	57,805124000	12,075005000	Sjö
			2021-07-20	N	=	50	=	70	Viss molnighet	Tjänligt	Ingen blomning	22,3	57,805124000	12,075005000	Sjö
			2021-06-29	N	=	10	=	30	Viss molnighet	Tjänligt	Ingen blomning	20,6	57,805124000	12,075005000	Sjö
			2021-06-08	N	=	30	<	10	Mulet	Tjänligt	Ingen uppgift	20,0	57,805124000	12,075005000	Sjö
		Bergsjön	2021-08-10	J	=	60	=	40	Mulet	Tjänligt	Ingen uppgift	19,3	57,749508373	12,064385903	Sjö
			2021-07-20	J	=	60	=	20	Mulet	Tjänligt	Ingen blomning	22,9	57,749508373	12,064385903	Sjö
			2021-06-29	J	=	50	=	50	Viss molnighet	Tjänligt	Ingen blomning	21,1	57,749508373	12,064385903	Sjö
			2021-06-08	J	=	7	=	2	Mulet	Tjänligt	Ingen uppgift	19,1	57,749508373	12,064385903	Sjö
		Stora Delsjön	2021-08-10	J	<	10	=	20	Regn	Tjänligt	Ingen uppgift	19,4	57,688709403	12,035173176	Sjö
			2021-07-20	J	=	30	=	20	Viss molnighet	Tjänligt	Ingen blomning	22,5	57,688709403	12,035173176	Sjö
			2021-06-29	J	=	50	=	10	Viss molnighet	Tjänligt	Ingen blomning	21,2	57,688709403	12,035173176	Sjö
			2021-06-08	J	=	22	=	5	Viss molnighet	Tjänligt	Ingen uppgift	19,4	57,688709403	12,035173176	Sjö
	Lerum	Aspen	2021-08-16	J	=	19	=	39	Viss molnighet	Tjänligt	Ingen blomning	19,5	57,767528835	12,252680322	Sjö
			2021-08-12	J	=	55	=	44	Viss molnighet	Tjänligt	Ingen blomning	20,1	57,767528835	12,252680322	Sjö
			2021-08-09	J	=	141	=	115	Viss molnighet	Tjänligt m. Anm.	Ingen blomning	20,6	57,767528835	12,252680322	Sjö
			2021-07-20	J	=	20	=	6	Viss molnighet	Tjänligt	Ingen blomning	22,6	57,767528835	12,252680322	Sjö
			2021-06-28	J	=	31	<	3	Klart	Tjänligt	Ingen blomning	20,7	57,767528835	12,252680322	Sjö
			2021-06-10	J	=	88	<	4	Klart	Tjänligt	Ingen blomning	18,3	57,767528835	12,252680322	Sjö
			2021-06-07	J	=	125	=	10	Regn	Tjänligt m. Anm.	Ingen blomning	20,5	57,767528835	12,252680322	Sjö
	Mölndal	Rådasjön	2021-08-16	J	=	2	=	20	Mulet	Tjänligt	Ingen blomning	20,2	57,663280401	12,059043866	Sjö

Rader 1 - 25

Kommun är lika med **Göteborg** ; **Härryda** ; **Lerum** ; **Mölndal** ; **Partille**
och År är lika med **2021**
och Län är lika med **Västra Götalands**
och Badplats är lika med **Aspen** ; **Bergsjön** ; **Kikås långvatten** ; **Kåsjön, barnbryggan** ; **Rådasjön** ; **Stensjön, strandpromenaden** ; **Stora Delsjön** ; **Stora Mölnesjön** ; **Tulebosjön**

Datum: 2022-04-07

Län	Kommun	Badplats	Datum	Eu-bad	*E. coli* prefix	*E. coli*	Intestinala enterokocker prefix	Intestinala enterokocker	Vädertyp	Kvalitet	Algblomning	Vattentemperatur	Latitud	Longitud	Badplatstyp
Västra Götalands	Mölndal	Rådasjön	2021-07-26	J	=	7	=	50	Viss molnighet	Tjänligt	Ingen blomning	24,6	57,663280401	12,059043866	Sjö
			2021-07-05	J	=	12	=	10	Viss molnighet	Tjänligt	Ingen blomning	24,4	57,663280401	12,059043866	Sjö
			2021-06-14	J	<	1	=	10	Klart	Tjänligt	Ingen blomning	19,3	57,663280401	12,059043866	Sjö
		Tulebosjön	2021-08-16	J	=	1	<	10	Mulet	Tjänligt	Ingen blomning	19,5	57,608766000	12,081882000	Sjö
			2021-07-26	J	=	3	<	10	Viss molnighet	Tjänligt	Ingen blomning	24,8	57,608766000	12,081882000	Sjö
			2021-07-05	J	=	10	<	10	Viss molnighet	Tjänligt	Ingen blomning	24,9	57,608766000	12,081882000	Sjö
			2021-06-14	J	=	14	<	10	Klart	Tjänligt	Ingen blomning	19,8	57,608766000	12,081882000	Sjö
		Stensjön, strandpromenaden	2021-08-16	N	=	33	=	80	Viss molnighet	Tjänligt	Ingen blomning	20,3	57,657665000	12,043631000	Sjö
			2021-07-26	N	=	20	<	30	Viss molnighet	Tjänligt	Ingen blomning	24,7	57,657665000	12,043631000	Sjö
						27	<	10	Viss molnighet	Tjänligt	Ingen blomning	24,7	57,657665000	12,043631000	Sjö
			2021-07-15	N	=	41	=	60	Klart	Tjänligt	Ingen blomning	27,5	57,657665000	12,043631000	Sjö
			2021-07-05	N	=	24	=	80	Viss molnighet	Tjänligt	Ingen blomning	24,5	57,657665000	12,043631000	Sjö
			2021-06-21	N	=	82	=	110	Viss molnighet	Tjänligt m. Anm.	Ingen blomning	22,1	57,657665000	12,043631000	Sjö
			2021-06-17	N	=	11	<	10	Klart	Tjänligt	Ingen blomning	20,4	57,657665000	12,043631000	Sjö
			2021-06-14	N	=	687	=	120	Klart	Tjänligt m. Anm.	Ingen blomning	19,9	57,657665000	12,043631000	Sjö
		Kikås långvatten	2021-08-16	N	=	11	=	50	Regn	Tjänligt	Ingen blomning	20,4	57,649565000	12,053892000	Sjö
			2021-07-26	N	=	27	<	10	Klart	Tjänligt	Ingen blomning	24,7	57,649565000	12,053892000	Sjö
			2021-07-05	N	=	3	<	10	Viss molnighet	Tjänligt	Ingen blomning	23,6	57,649565000	12,053892000	Sjö
			2021-06-14	N	=	2	<	10	Klart	Tjänligt	Ingen blomning	19,8	57,649565000	12,053892000	Sjö
	Partille	Kåsjön, barnbryggan	2021-08-03	J	=	6	<	10	Ingen uppgift	Tjänligt	Ingen uppgift	19,0	57,711640804	12,142767432	Sjö
			2021-07-13	J	=	28	=	60	Saknas	Tjänligt	Ingen uppgift	22,0	57,711640804	12,142767432	Sjö
			2021-06-29	J	=	44	=	90	Saknas	Tjänligt	Ingen uppgift	20,0	57,711640804	12,142767432	Sjö
			2021-06-10	J	=	36	=	20	Saknas	Tjänligt	Ingen uppgift	19,0	57,711640804	12,142767432	Sjö

Rader 26 - 48 (slut)

Datum: 2022-04-07

Kommun är lika med **Göteborg** ; **Härryda** ; **Lerum** ; **Mölndal** ; **Partille**
och År är lika med **2021**
och Län är lika med **Västra Götalands**
och Badplats är lika med **Aspen** ; **Bergsjön** ; **Kikås långvatten** ; **Kåsjön, barnbryggan** ; **Rådasjön** ; **Stensjön, strandpromenaden** ; **Stora Delsjön** ; **Stora Mölnesjön** ; **Tulebosjön**