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Dissemination of antibiotic resistance genes in a Nile Tilapia cage culture, 2017

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ABSTRACT

Antibiotic resistance is one of the world most threatening health problems. It results from unnecessary and inappropriate use of antibiotics, which to a greater part is caused by excessive amounts of antibiotics being used in husbandry, mostly for preventive measures. The effects of antibiotics on bacterial resistances in the environment is a matter of increasing concern and is largely unknown. In addition, little information is available about how the use of antibiotics in farming systems can be managed in a manner that has negligible effects on the natural microbiome of the environment. In this study, we established the dynamics of antibiotic resistance in a caging fish farm in the Taasan-Bangpla canal, Kamphaeng Saen, in January 2018. Water samples were collected in the fishing-cage, as well as at upstream and downstream sites, for a comparison of the occurrence of antibiotic resistance genes through metagenome analysis. The results demonstrated that the antibiotic resistance genes in the downstream sample were different and occurred at lower frequency than those in the fish cage, suggesting that the fish farming did not spread any sources of antibiotic resistance to the surface of the canal. Our observations were explained by the high-water flow in the canal, 36.47 x 10^6 m^3/month, during the period of our study and/or the absence of active antibiotics residues downstream. Future studies will require additional analyses under various conditions and different periods in the year.

Keywords: Antibiotic resistance, Metagenomics, Fish farm, Bacteria, Water quality

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บทคัดย่อ

แบคทีเรียดื้อยาปฏิชีวนะเป็นปัญหาในระดับโลก สาเหตุการเพิ่มขึ้นมีความสัมพันธ์กับปริมาณยาปฏิชีวนะที่ถูกใช้ดังนั้นจึงมีมาตรการลดการใช้ยาปฏิชีวนะโดยไม่จำเป็นและไม่ถูกต้อง โดยเฉพาะในปศุสัตว์ที่ใช้ยาปฏิชีวนะจำนวนมากในการป้องกัน อย่างไรก็ตามไม่มีข้อยืนยันชัดเจนว่าการใช้ในปริมาณเท่าใดและใช้อย่างไรจึงไม่ก่อให้เกิดปัญหาการเพิ่มขึ้นและแพร่กระจายเชื้อดื้อยาในสิ่งแวดล้อม ดังนั้นในการศึกษาเชิงประเมินนี้เราต้องการทราบว่าการเลี้ยงปลาในกระชังตามวิธีทั่วไปของเกษตรกรในคลองท่าสาร-บางปลา กำแพงแสน มีผลต่อการส่งเสริมและแพร่กระจายของยีนดื้อยาในคลองหรือไม่ โดยเปรียบเทียบความถี่ของยีนดื้อยาในน้ำ ณ ตำแหน่งต้นน้ำ ในกระชัง และ ท้ายกระชัง แต่ละจุดห่างกันประมาณ 800 เมตร ในเดือนมกราคม 2561 ด้วยวิธีเมตาจีโนมิกซ์ พบว่า ชนิดของยีนดื้อยาที่พบในกระชังปลา แตกต่างจากในตำแหน่งท้ายน้ำและความถี่ของยีนดื้อยาท้ายน้ำมีปริมาณต่ำมาก สันนิษฐานว่าการเลี้ยงปลาในกระชังของเกษตรกรที่ศึกษาไม่ส่งผลต่อการเพิ่มขึ้นและแพร่กระจายของยีนดื้อยาในท้ายน้ำเพราะอาจไม่มียาปฏิชีวนะตกค้างในท้ายน้ำและ/หรือระยะเวลา ที่ศึกษาเป็นช่วงที่มีการระบายน้ำลงในคลองท่าสาร-บางปลาในปริมาณสูงขึ้นคือ 36.47 ล้านลูกบาศก์เมตร/เดือน ข้อเสนอแนะจากผลการศึกษาคือ ควรมีการศึกษาเพิ่มเติมตลอดช่วงปีและวิเคราะห์ผลจากกิจกรรมที่เกิดขึ้นในตำแหน่งต่าง ๆ ของคลอง

คำสำคัญ: การดื้อยาปฏิชีวนะ เมตาจีโนมิกส์ ฟาร์มปลา แบคทีเรีย คุณภาพน้ำ

Introduction

One of the most threatening challenges to human health is the increasing antimicrobial resistance that affects all continents of the world. In the report of the World Economic Forum it is estimated that in 2050 the global risk of antimicrobial resistance annual death toll is estimated to reach 10 million/year and cost 1-1-3.8% of global GDP (World Economic Forum, 2018: online). This ominous situation results from the people's ignorance to use abundant amounts of antibiotics, of which medication is partly ineffective or unnecessary. Every treatment has the small risk that few bacteria in a population acquire resistant to the drug, a risk that increases dramatically when more often and longer particular antibiotic treatments are prescribed. Until now, medical practitioners and pharmacists have underestimated the risk to this problem, leading to a dramatic and perilous risk of multiple antimicrobial resistances. An even more serious cause to antimicrobial resistance comes from husbandry and aquaculture activities, where, since the 50s, large amounts of antibiotics are used for disease prevention, creating animal food which becomes key reservoirs of antibiotic resistance bacteria (Founou *et al.*, 2016). Antimicrobial resistance that often occurs in animal farms can eventually spread to humans through the food chain and direct contact.

The uncontrolled increase in the prevalence of antibiotic-resistant pathogens creates a world in which less and less antimicrobial agents remain to treat infections. The estimate is that by 2050, there will be no effective antibiotic available, if no new drug is developed or discovered (Vivas *et al.* 2019). Being aware of these serious risks have encouraged international to restrict or ban antimicrobials used for animals, although there is little proof that antimicrobial resistance in

human came from animal sources. Xiong *et al.* (2018) described in their review the history and trends of antimicrobial use, the emergence and spread of antimicrobial resistance in food animals, and gave suggestions how spread of antimicrobial resistance can be kept under control. Oloso *et al.* (2018) reviewed and evaluated previous studies for their contributions on food animals and the environment to the antimicrobial resistance burden in Nigeria. Almost all papers mentioned multidrug resistance of which 18 bacterial spp. were found resistant to various locally available antimicrobials. The studies on drug residues reported that the levels of residues were above the recommended international limit. The high amounts of residues and antimicrobials released into the environment were kept responsible for sustaining the antimicrobial resistance pool, and so caused potential risk to the public health.

Much attention was paid to the dramatic consequences of exponentially increased antibiotic resistance in fish farming areas in recent decades. An increasing number of resistant bacteria followed that enormous use or misuse of antibiotics to prevent possible diseases and overcome major production problems (reviewed in Caruso, 2016). In an extensive study on catfish and tilapia aquaculture in the Ashanti Region of Ghana, Agoba *et al.* (2017) assessed whether the fish farms contributed to antibiotic resistances. Although the majority of the farmers declared not to use antibiotics on their farms, observed antibiotic resistance may have been explained from untreated waste disposal and from poultry manure from commercial poultry farms as source of nutrition in their ponds. The antibiotics that were administered through the undigested fish feed may leak into the environment and accumulate, resulting in resistance (Miranda *et al.*, 2018). Santos & Ramos (2018) also warned that passage of antimicrobial resistance genes and resistant bacteria from aquatic to terrestrial animal husbandry and to the human environment and *vice versa* were also potentially very harmful to both humans and animals. They strongly pleaded for global joint efforts to reduce the excessive use of such antimicrobial reagents and to support stakeholders to implement other disease-prevention measures.

The public menace of excessive use of antibiotics in Thailand's health organizations and agricultural activities has been widely discussed in recently published reports (Ministry of Public Health, Thailand, 2017; Sumpradit *et al.* 2017; Chanvatik *et al*. 2019), providing general recommendations and national strategy aiming at the control of antimicrobial resistance issues, including the country's commitment to join global initiatives in resolving antimicrobial resistance by reducing antimicrobial resistance morbidity by 50%. The proposed goals included dramatic reductions of antimicrobial use for human health, feed animal production and companion animals; antimicrobial resistance surveillance system; regulation of antimicrobial distribution; and above all, public knowledge on antimicrobial resistance and awareness of professional use of antimicrobials.

As only a few of the unknown bacteria in the environment can be grown on standard media under controlled laboratory conditions, studies of antibiotic resistances in infectious

bacteria requires the development of culture independent technologies, such as sequence-based metagenomics (Schmieder & Edwards, 2012). Boolchandani *et al.* (2019) presented a detailed overview of antimicrobial resistance identification and characterization methods, including traditional antimicrobial susceptibility testing to recent deep-learning methods. Crofts *et al.* (2017) described the important work in this resistome field, including recent scientific advances, and proposed a resistome strategy for identification and alleviation of upcoming antibiotic resistance threats. Specifically, in the field of fishery and aquaculture, comprehensive studies of bacterial communities, the abundance and diversity of antibiotic resistance genes (ARGs), and mobile genetic elements were carried out for herbicides polluted soils (Lou *et al*., 2019), global freshwater lakes, rivers and reservoirs (Jiang *et al*. 2018; Liu *et al*., 2018; Yang *et al*., 2018) and various freshwater aquaculture environments (Wang *et al*., 2018; Fang *et al*., 2019).

In the present study, we evaluated a protocol for metagenome analysis of ABRs. Water samples were collected from red tilapia cages in Taasan-Bangpla canal, in Kamphaeng Saen, Nakhon Pathom province. This 66 km long canal extends from Kanchanaburi to Nakhon Pathom and is used for water diversion from the Mae-Klong river to the Tha-Chin river. We examined the contribution of this fish farm to the ARG in the downstream environment. In addition, we compared the results the ARG frequency at the fish farm with an 800s m site upstream of the farm and 800s m downstream. We hypothesized that fish culture may contribute ARGs to some extend in the canal, while the ARG selection pressure for the downstream may rely on a combination of unknown factors.

Materials and Methods

Water sample sites

We selected three sites with relatively low anthropogenic effects in Taasan-Bangpla canal. We first chose a cage fish farm of red hybrid Nile tilapia (Oreochromis spp.), which was located a few km upstream of a small community. The second and third sites were 800s meter upstream and downstream from the farm, respectively (Figure 1, Table 2). The farm held 15 cages with 2,000 fishes per cage. For fish feeding, commercial dry food was used, without antibiotic, according to the farmer's information. The water samples were collected in January, 2018, with the water flow rate of 36.47 x 106 m3/month (Figure 2). At each site, three samples of 2 L water each were collected, at a depth of about 20 cm; and pooled them together in a 5 L flask on ice. The samples were transferred to the laboratory for immediate DNA isolation.

Figure 1 The sites of water samples in the Taasan-Bangpla canal, Kampang Saen, Nakhorn Pathom (Google, 2019); 1. Upstream, 2. Fish cage farm and 3. Downstream, each on 800 meters distance

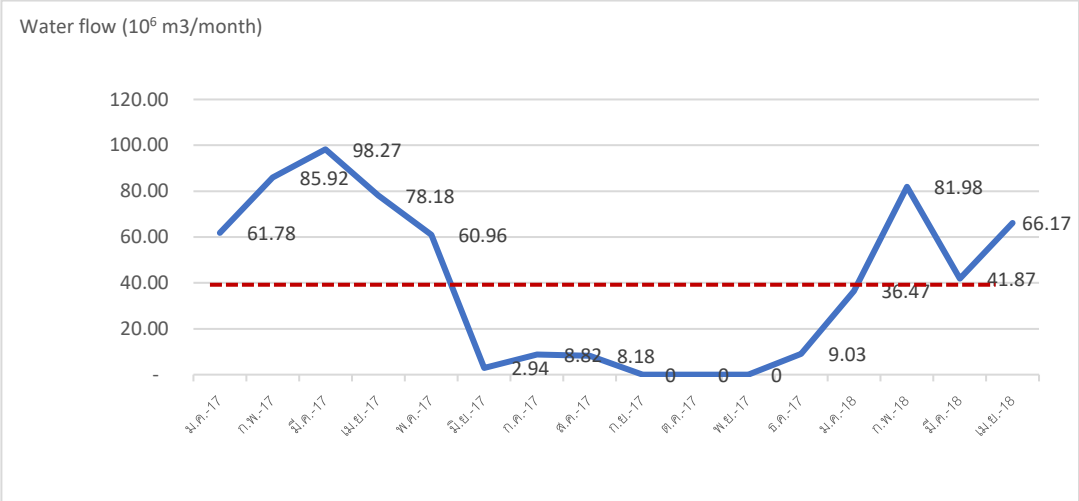


Figure 2 Water flow rate (x 10^6 m^3/month) during January 2017-April 2018, in the Taasan-Bangpla canal (Nakhorn Pathom Provincial Irrigation Office, July 2018, pers. comm.). The red line indicates the threshold of good water quality; above the line is high, below the line is low quality (Ingthamjitr *et al*., 2017).

DNA isolation

The water samples were first filtered through Whatman Filter paper grade 1, in order to remove residues and then filtered through a 2-µm nylon filter to isolate the bacteria. The filters were cut into small pieces and were soaked in 10 mL normal saline, followed by harvesting the cells after a 5 minutes 13,000 rpm bench-top centrifugation. The pellets were dissolved in 1 mL normal saline for the DNA isolation using the Norgen water RNA/DNA purification kit (Norgen Biotek Corp., Ontario, Canada) following the company's instruction. The isolated DNA was dissolved in TE for quality and quantity check through NanoDrop (Thermo Fisher Scientific Inc.), Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and 1% agarose gel. The DNA library

preparation was constructed following the manufacturer's protocol (VAHTS universal DNA library Prep Kit for Illumina). Two microgram DNA was sheared to <500 bp by sonication (CovarisS220), followed by adding the adaptors to both ends. Size selection of adaptor-ligated DNA was performed and fragments of ~410 bp (with the insert size of ~350 bp) were collected. The DNA fragments were amplified by PCR using P5 and P7 primers, with both primers can anneal to perform bridge PCR. P7 primer contains a six-base index allowing for multiplexing. The PCR products were cleaned up and quantified by Qubit2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

DNA sequencing

The DNA libraries were sequenced on the Illumina HiSeq (Illumina, San Diego, CA, USA), using 2 x 150 paired-end configuration, with 5.0-6.0 Gb data per sample.

Metagenomics analysis

We used MGmapper version PE_2.24 (Petersen *et al.*, 2017), which utilized BWA-mem version 0.7.17-r1188 (Li, 2013) and SAMtools version 1.6 (Li *et al.*, 2009), as a bioinformatic pipeline to process our data. Using MGmapper, the adapter sequences were removed from the raw paired reads with cutadapt version 1.15 (Martin, 2011), and the minimum read length after trimming (-m) was set to 30. The minimum Quality q cutoff (-q) was set to 30, and the QUALITY_BASE values (-B) was set to 33. Next, the trimmed read pairs were mapped against the PhiX database, using BWA-mem, to remove any internal Illumina control reads. The filtered read pairs after this step were called notPhiX, which were set to 100% to calculate the R_abundance.Then, MGmapper mapped the filtered read pairs onto the databases in Table 1 using BWA-mem. The minimum alignment score (-A) was set to 30, and the fraction of matches+mismatches (-t) was set to 0.8. The match counts and their quality scores on each entry in each database were collected and summarized. The filtered read pairs were mapped against the Bacteria, Bacteria draft, Virus, Fungi, and Protozoa databases in the Best mode, which chose only one best database match for each read pair. The same read pairs were also mapped against the MetaHitassembly (Nielsen et al., 2014), ResFinder (Zankari et al., 2012), and Plasmid databases using the Full mode, in which each read pair can be mapped onto multiple databases. The Bacteria and the Bacteria draft databases were generated from the bacteria and bacteria draft sequences downloaded from NCBI using MGmapper_makedb.pl on May 8th, 2018.The rest of the databases were downloaded from www.cbs.dtu.dk/public/MG mapper/databases/, which were dated in June 2015. The top 20 most abundant hits were selected for further analysis without using the post-processing filter function provided with MGmapper.The minimum size normalized abundance (-r), minimum unique reads ratio (-D), and the minimum read count (-U) were set to 0. The maximum mismatched ratio (-L) was set to 1.

Normalized abundance estimation

The raw read numbers were normalized by the total number of reads after cleaning process (notPhiX) for the abundance of bacteria, plasmid, human gut microbes and ARGs. The normalized reads by the sum numbers of bacteria and bacteria draft were

used to compare the frequency of plasmid, human gut microbes and ARGs in each sample

Table 1 Reference database used to map three datasets of water sources studied

Databases	Reference	link
Bacteria	(Petersen *et al*., 2017)	--
Bacteria draft	(Petersen *et al*., 2017)	--
Virus	(Petersen *et al*., 2017)	http://www.cbs.dtu.dk/public/MGmapper/databases/Virus.gz
Fungi	(Petersen *et al*., 2017)	http://www.cbs.dtu.dk/public/MGmapper/databases/Fungi.gz
Protozoa	(Petersen *et al*., 2017)	http://www.cbs.dtu.dk/public/MGmapper/databases/Protozoa.gz
MetaHitassembly	(Nielsen *et al*., 2014)	http://www.cbs.dtu.dk/public/MGmapper/databases/MetaHitAssembly.gz
ResFinder	(Zankari *et al*., 2012)	http://www.cbs.dtu.dk/public/MGmapper/databases/ResFinder.gz
Plasmid	(Petersen *et al*., 2017)	http://www.cbs.dtu.dk/public/MGmapper/databases/Plasmid.gz

Note: *Bacteria and Bacteria draft database were downloaded using *MGmapper_makedb.pl*, which is a part of the MGmapper package on May 8th, 2018.

Results

Sequencing reads

The DNA datasets from the three sample sites, i.e., upstream (T1), fish cage (T2) and downstream (T3) of the cage-fish farm were generated through paired-end Illumina sequencing, amounting 5.7 to 6.0 Gigabytes (Table 2). The average number of analyzed reads were 37 x 106 reads, of which 8.50, 4.95 and 4.52 % in T1, T2 and T3, respectively, were assigned to bacteria (Table 3). The T1 site which was close to a household animal farm nearby, might have affected the bacterial load in the canal. In addition, the reads hit to human intestine tracts bacteria were higher in T1 (0.021%) than in T2 and T3 (0.001%), which pointed at a farm nearby (Table 3). The percentage of plasmids assigned were 0.093, 0.103, 0.089, while the ARGs hits were 8.7 x 10-4, 1.7 x 10-4 and 0.05 x 10-4 in T1, T2, and T3, respectively (Table 3).

Bacteria

The most abundant bacteria among the three sites were soil and aqua bacteria. The total frequency of bacteria in T1 was higher than in T2 and T3. The six most prevalent strains in T1 were missing in the T2 and T3 samples, while other strains, like Polynucleobacter necessarius subsp. asymbioticus QLW-P1DMWA-1, which were found in all sites occurred at almost equal frequencies (Figure 3). The strains that were more common in T2 and T3 were relative rare in T1, although the rarest strains that we detected in all sites likely represented endemic strains that were under low selective pressure. However, few strains, like Flavobacterium indicum GPTSA100-9 (gi|380500974|emb|HE774682.1|) were more specific in T2 and T3 than T1.

Metagenomic Human Intestine Tract Microbiota

The source of ARGs in farm is animal discharges including feces. Therefore, we are interested in evaluating the gut microbials from animal feces for how they are relevant to ARG prevalence. Unfortunately, the farm animal gut microbiota database is not available, then the closest database was chosen to be reference. According to the gut microbiota among animals is diverse in an ecophylogenetic and evolutionary clocklike manner (Gaulke *et al.*, 2018), then the human gut microbiome database, Meta Hit Assembly was used to be the reference of mammal farm animal microbiota.

The datasets were mapped to the MetaHitassembly database in order to search for human gut microbes. We observed higher values in T1 than in T2 and T3 (Table 4). The most prevalence strains in T1 were Sutterella sp. MGS:521, Escherichia coli MGS:4, Sutterella wadsworthensis MGS:135 and Proteobacteria bacterium MGS:139, and these strains were also found in T2 and T3 at lower frequency, but still in the list of top ten strains (Figure 4).

Antibiotic resistance gene

Normalized frequency of ARGs as part of the total number of bacteria and bacterial draft of T1, T2 and T3 were 0.1021, 0.0033 and 0.0001 % (Table 4). In T1 the most prevalent ARGs were specific to quinolone, beta-lactam, phenicol, tetracycline and trimethoprim. In contrast, the ARGs found in T2 were specific to aminoglycoside, tetracycline, phenicol, beta-lactam and sulphonamide, while in T3 we found only beta-lactam resistances (Figure 5). We also observed examples of resistance to the same antibiotic classes in the three sites, that displayed different ARGs. Some ARGs, like QnrS2_1_JF261185 (quinolone Resistance), QnrS6_1_HQ631376 (quinolone Resistance), POM-1_1_GU002295(Beta-lactam resistance)—which were more frequent in T1, but less in T2 and T3—may indicate selection from the household farm residues. Most of the top rank ARGs of T2 were not found in T1 and T3 suggesting some specificity to that particular site. In contract to T1 and T2, only one ARG was found in T3 at very low frequency, pointing at the absence of any antibiotic selective pressure in this downstream site.

Plasmids

Normalized frequencies of plasmid in T1, T2 and T3 were 1.08, 2.08 and 1.94 %, respectively (Table 4). The most abundant plasmid in the three samples were found less different when compared to their ARGs. The Serratia liquefaciens ATCC 27592 plasmid was detected in all samples with the highest frequency in T1, while T2 and T3 share many more plasmids than with T1 suggesting that the T1 environment has a stronger selection pressure than T2 and T3 (Figure 6). Furthermore, we found that the majority of the reference sequence hits were transposable element repeats indicating a strong direct selection of these mobile elements.

Table 2 The description of the sample sites in the Taasan-Bangpla canal and the number of base pair sequenced and reads of DNA samples

	Upstream (T1)	**Fish cage (T2)**	**Downstream (T3)**
Distance (meters)	-820	0	+840
GPS Coordinate	13.995070, 99.958070	13.996480, 99.962554	13.995658, 99.969696
Activity nearby /at the site	Household animal farm	Cage fish farm	Plantation
Number of base (Gbs)	5.8826607	5.7194277	5.9765442
Number of paired reads	19,608,869	19,064,759	19,921,814
Analyzed reads mapped	37,753,060	36,919,446	38,659,270

Table 3 The number and percentage of the reads mapped to reference databases

Reference database	**The number of read mapped**		
	Upstream (T1)	**Fish cage (T2)**	**Downstream (T3)**
NotPhiX	37,753,060 (100%)	36,919,446 (100%)	38,659,270 (100%)
ResFinder	328 (0.001%)	62 (0.000%)	2 (0.000%)
Plasmid	35,064 (0.093%)	38,116 (0.103%)	34,222 (0.089%)
MetaHitassembly	7,754 (0.021%)	468 (0.001%)	322 (0.001%)
Bacteria	2,453,368 (6.498%)	1,224,298 (3.316%)	1,263,954 (3.269%)
Bacteria draft	759,044 (2.011%)	604,048 (1.636%)	485,128 (1.250%)

Table 4 Normalized abundance of reads by total number of reads mapped to bacteria and bacteria draft database

Reference database	**Percentage (%) of reads mapped to databases**		
	Upstream (T1)	**Fish cage (T2)**	**Downstream (T3)**
ResFinder	0.102	0.003	0.0001
Plasmid	1.08	2.08	1.94
MetaHitassembly	0.24	0.02	0.00
Bacteria	75.79	66.18	71.57
Bacteria Draft	23.45	32.65	27.36

Bacterial strain	Reference sequence	T1	T2	T3
Aeromonas veronii B565, complete genome	gi|328802836|gb|CP002607.1|	1.151	0.007	0.004
Pseudomonas mendocina NK-01, complete genome	gi|328915200|gb|CP002620.1|	0.734	0.006	0.001
Aeromonas hydrophila subsp. hydrophila ATCC 7966, complete genome	gi|117558854|gb|CP000462.1|	0.716	0.008	0.001
Aeromonas hydrophila ML09-119, complete genome	gi|507219248|gb|CP005966.1|	0.68	0.005	0.001
Shewanella sp. ANA-3 chromosome 1, complete sequence	gi|117610791|gb|CP000469.1|	0.628	0	0
Shewanella sp. MR-4, complete genome	gi|117610791|gb|CP000469.1|	0.439	0	0
Pseudomonas mendocina ymp, complete genome	gi|145573243|gb|CP000680.1|	0.434	0.012	0.002
Shewanella sp. MR-7, complete genome	gi|113886955|gb|CP000444.1|	0.378	0	0
Polynucleobacter necessarius subsp. necessarius STIR1, complete genome	gi|171192370|gb|CP001010.1|	0.359	0.302	0.335
Polynucleobacter necessarius subsp. asymbioticus QLW-P1DMWA-1, complete genome	gi|145046595|gb|CP000655.1|	0.298	0.248	0.267
Shewanella oneidensis MR-1, complete genome	gi|410519462|gb|AE014299.2|	0.273	0	0
Stenotrophomonas maltophilia K279a complete genome, strain K279a	gi|190010013|emb|AM743169.1|	0.244	0.001	0.002
Beta proteobacterium CB, complete genome	gi|455439634|gb|CP004348.1|	0.243	0.209	0.187
Shewanella sp. MR-7, complete sequence	gi|113890962|gb|CP000445.1|	0.215	0	0
Flavobacterium indicum GPTSA100-9 complete genome	gi|380500974|emb|HE774682.1|	0.214	0.963	1.741
Stenotrophomonas maltophilia JV3, complete genome	gi|343776783|gb|CP002986.1|	0.191	0.001	0.002
Stenotrophomonas maltophilia D457 complete genome	gi|384076029|emb|HE798556.1|	0.187	0.001	0.002
Pseudomonas resinovorans NBRC 106553 DNA, complete geonome	gi|512374267|dbj|AP013068.1|	0.182	0.008	0.004
Aeromonas hydrophila 4AK4, complete genome	gi|569545899|gb|CP006579.1|	0.17	0.002	0
Acinetobacter calcoaceticus PHEA-2, complete genome	gi|325121063|gb|CP002177.1|	0.155	0.008	0.004
Emticicia oligotrophica DSM 17448, complete genome	gi|387853393|gb|CP002961.1|	0.035	0.461	0.015
Novosphingobium aromaticivorans DSM 12444, complete genome	gi|87133707|gb|CP000248.1|	0.052	0.285	0.325
Acidovorax sp. KKS102, complete genome	gi|407894523|gb|CP003872.1|	0.143	0.239	0.148
Ramlibacter tataouinensis TTB310, complete genome	gi|334728683|gb|CP000245.1|	0.101	0.188	0.13
Leptothrix cholodnii SP-6, complete genome	gi|170774137|gb|CP001013.1|	0.045	0.141	0.053
Dechloromonas aromatica RCB, complete genome	gi|71845263|gb|CP000089.1|	0.065	0.112	0.02
Acidovorax sp. JS42, complete genome	gi|120604516|gb|CP000539.1|	0.042	0.1	0.098
Rubrivivax gelatinosus IL144 DNA, complete genome	gi|381376528|dbj|AP012320.1|	0.097	0.097	0.031
Agrobacterium tumefaciens str. C58 circular chromosome, complete sequence	gi|159139455|gb|AE007869.2|	0.046	0.096	0.055
Acidovorax ebreus TPSY, complete genome	gi|221728669|gb|CP001392.1|	0.043	0.093	0.064
Novosphingobium sp. PP1Y main chromosome, complete replicon	gi|333937619|emb|FR856862.1|	0.02	0.092	0.1
Agrobacterium tumefaciens str. C58 linear chromosome, complete sequence	gi|159140696|gb|AE007870.2|	0.042	0.084	0.053
Acidovorax citrulli AAC00-1, complete genome	gi|120587178|gb|CP000512.1|	0.039	0.081	0.058
Acidovorax avenae subsp. avenae ATCC 19860, complete genome	gi|323371659|gb|CP002521.1|	0.038	0.08	0.055
Variovorax paradoxus S110 chromosome 1, complete sequence	gi|239799596|gb|CP001635.1|	0.032	0.064	0.046
Alicycliphilus denitrificans K601, complete genome	gi|329308025|gb|CP002657.1|	0.026	0.056	0.04
Burkholderia xenovorans LB400 chromosome 3, complete sequence	gi|91692731|gb|CP000272.1|	0.003	0.016	0.045
Sphingopyxis alaskensis RB2256, complete genome	gi|98975575|gb|CP000356.1|	0.007	0.035	0.041
Flavobacterium branchiophilum FL-15, complete genome	gi|345528129|emb|FQ859183.1|	0.006	0.038	0.041
Burkholderia multivorans ATCC 17616 genomic DNA, complete genome, chromosome 3	gi|189338131|dbj|AP009387.1|	0.003	0.016	0.04

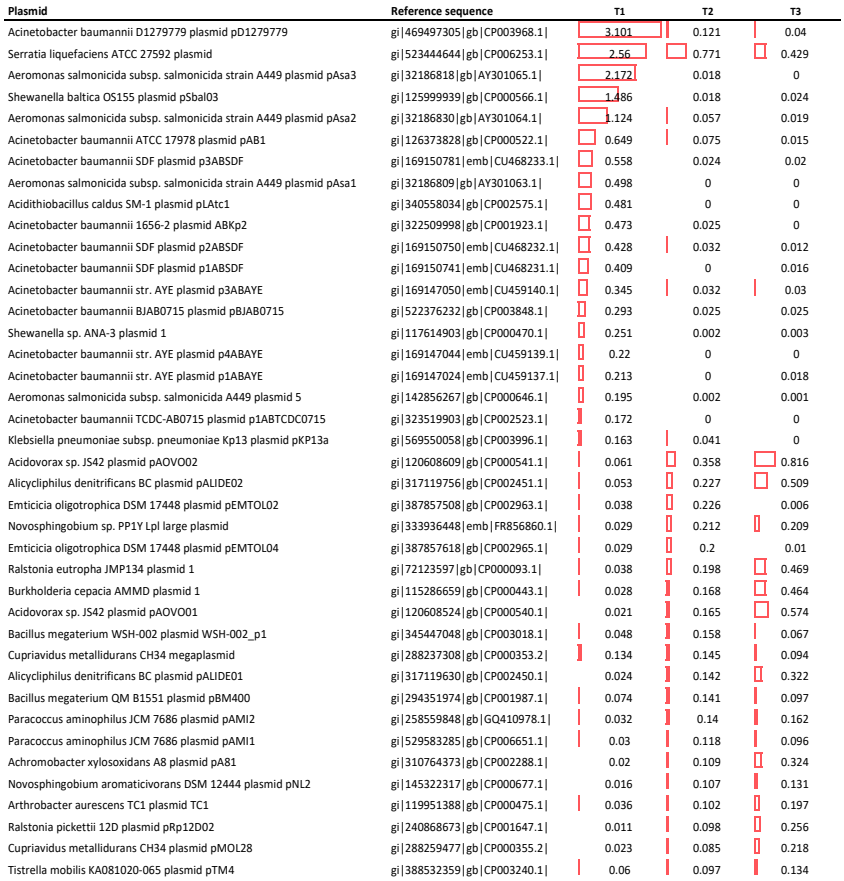
Figure 3 Heatmap of the top rank bacteria and their sequence abundance of the datasets of the water samples (T1 is Upstream site, T2 is fish cage farm site and T3 is downstream site; sequence abundance showing size normalized read count abundance and equals the number of read mapped to reference sequence x 100 / (2 x reference size)

Metahit assembly	Scaffold	T1	T2	T3
Sutterella sp.MGS:521	MGS521_scaffold4	28.066	0.943	0.629
Escherichia coli MGS:4	MGS4_scaffold1	21.42	0	0.319
Sutterella sp. MGS:521	MGS521_scaffold9	16.436	1.188	0.198
Sutterella wadsworthensis MGS:135	MGS135_scaffold72	13.4	0.993	0.372
Proteobacteria bacterium	MGS139_scaffold11	4.219	0.469	0.312
Succinatimonas sp. MGS:777	MGS777_scaffold4	3.661	0.359	0
Sutterella sp. MGS:351	MGS351_scaffold109	2.978	0.313	0.157
Parasutterella excrementihominis	MGS233_scaffold13	2.406	0.314	0.105
Azospirillum sp.	MGS260_scaffold73	1.618	0.319	0.147
Sutterella wadsworthensis MGS:135	MGS135_scaffold122	1.211	0.173	0.346
Odoribacter splanchnicus	MGS14_scaffold1	1.098	0	0.183
Collinsella sp.	MGS166_scaffold9	1.002	0.472	0.177
Coprobacillus sp.	MGS235_scaffold7	0.968	0	0.138
Brachyspira sp.	MGS700_scaffold192	0.802	0.178	0.089
Proteobacteria bacterium MGS:495	MGS495_scaffold63	0.668	0.223	0
Succinatimonas sp. MGS:777	MGS777_scaffold52	0.58	0	0
Collinsella sp. MGS:166	MGS166_scaffold5	0.56	0	0
Sutterella sp. MGS:351	MGS351_scaffold108	0.521	0	0.104
Klebsiella variicola	MGS634_scaffold126	0.309	0.039	0
Bacteroides sp.	MGS633_scaffold210	0.307	0	0
Azospirillum sp. MGS:239	MGS239_scaffold22	0	0.434	0
Dialister sp. MGS:588	MGS588_scaffold4	0	0.13	0
Bacteroides sp. MGS:770	MGS770_scaffold25	0.083	0.083	0
Acetobacter sp. MGS:267	MGS267_scaffold12	0.178	0.071	0.036
Coprobacillus sp. MGS:235	MGS235_scaffold16	0.048	0.048	0
Sutterella sp. MGS:351	MGS351_scaffold13	0.02	0.039	0
Erysipelotrichaceae bacterium MGS:64	MGS64_scaffold48	0	0	0.227
Eubacterium siraeum MGS:80	MGS80_scaffold134	0.181	0	0.181
Streptococcus salivarius MGS:79	MGS79_scaffold35	0	0	0.18
Sutterella sp. MGS:521	MGS521_scaffold5	0.003	0.003	0.139
Bacteroides sp. MGS:144	MGS144_scaffold3	0	0	0.095
Clostridium sp. MGS:678	MGS678_scaffold11	0	0	0.093

Figure 4 Heatmap of the top rank MetaHitassembly and their sequence abundance in the datasets of the three water samples

Antibiotic resistance gene	Reference gene	T1	T2	T3
Quinolone resistance	QnrS2_1_JF261185	3.805	0	0
Quinolone resistance	QnrS6_1_HQ631376	0.913	0	0
Beta-lactam resistance	POM-1_1_GU002295	0.853	0	0
Phenicol resistance	catB1_1_M58472	0.635	0.159	0
Tetracycline resistance	tet(39)_2_EU495991	0.563	0	0
Trimethoprim resistance	dfrA31_1_AB200915	0.422	0	0
Tetracycline resistance	tet(39)_2_EU495989	0.422	0	0
Beta-lactam resistance	cphA7_1_AY227053	0.392	0	0
Beta-lactam resistance	ampH_1_AJ276031	0.377	0	0
Beta-lactam resistance	blaOXA-12_1_U10251	0.377	0	0
Beta-lactam resistance	blaOXA-204_1_JQ809466	0.376	0	0
Beta-lactam resistance	blaADC-25_1_EF016355	0.347	0	0
Beta-lactam resistance	blaMOX-6_1_GQ152601	0.347	0	0
Beta-lactam resistance	blaCEPH-A3_1_AY112998	0.261	0	0
Beta-lactam resistance	cphA8_1_AY261375	0.261	0	0
Aminoglycoside resistance	aadA1_3_JQ414041	0.253	0.126	0
Beta-lactam resistance	ampS_1_X80276	0.252	0	0
Beta-lactam resistance	ampH_2_HQ586946	0.252	0	0
Aminoglycoside resistance	aph(3_)-IIc_1_AM743169	0.246	0	0
Sulphonamide resistance	sul2_14_AJ514834	0.244	0	0
Aminoglycoside resistance	aadB_1_JN119852	0	0.375	0
Tetracycline resistance	tet(A)_3_AY196695	0.167	0.167	0
Aminoglycoside resistance	aadA2_1_X68227	0	0.128	0
Aminoglycoside resistance	aadA8b_1_AM040708	0	0.126	0
Beta-lactam resistance	blaOXA-20_1_AF024602	0	0.125	0
Beta-lactam resistance	blaOXA-5_1_X58272	0	0.124	0
Sulphonamide resistance	sul2_17_U57647	0	0.123	0
Sulphonamide resistance	sul2_6_FN995456	0	0.123	0
Beta-lactam resistance	blaOXA-209_1_JF268688	0	0.121	0
Beta-lactam resistance	blaOXA-3_1_L07945	0	0.121	0
Beta-lactam resistance	blaOXA-21_2_DQ993182	0.121	0.121	0
Sulphonamide resistance	sul1_7_FJ715937	0	0.119	0
Sulphonamide resistance	sul1_29_AJ746361	0	0.119	0
Sulphonamide resistance	sul1_20_JF262165	0	0.119	0
Sulphonamide resistance	sul1_30_JF262178		0.119	0
Sulphonamide resistance	sul1_22_AY115475		0.119	0
Sulphonamide resistance	sul1_24_EU117158		0.119	0
Sulphonamide resistance	sul3_5_AB281182	0	0.117	0
Beta-lactam resistance	blaTEM-155_1_DQ679961	0	0	0.116

Figure 5 Heatmap of the top rank antibiotic resistance genes and their sequence abundance of three datasets of water studied



Plasmid	Reference sequence	T1	T2	T3
Acinetobacter baumannii D1279779 plasmid pD1279779	gi|469497305|gb|CP003968.1|	3.101	0.121	0.04
Serratia liquefaciens ATCC 27592 plasmid	gi|523444644|gb|CP006253.1|	2.56	0.771	0.429
Aeromonas salmonicida subsp. salmonicida strain A449 plasmid pAsa3	gi|32186818|gb|AY301065.1|	2.172	0.018	0
Shewanella baltica OS155 plasmid pSbal03	gi|125999939|gb|CP000566.1|	1.486	0.018	0.024
Aeromonas salmonicida subsp. salmonicida strain A449 plasmid pAsa2	gi|32186830|gb|AY301064.1|	1.124	0.057	0.019
Acinetobacter baumannii ATCC 17978 plasmid pAB1	gi|126373828|gb|CP000522.1|	0.649	0.075	0.015
Acinetobacter baumannii SDF plasmid p3ABSDF	gi|169150781|emb|CU468233.1|	0.558	0.024	0.02
Aeromonas salmonicida subsp. salmonicida strain A449 plasmid pAsa1	gi|32186809|gb|AY301063.1|	0.498	0	0
Acidithiobacillus caldus SM-1 plasmid pLAtc1	gi|340558034|gb|CP002575.1|	0.481	0	0
Acinetobacter baumannii 1656-2 plasmid ABKp2	gi|322509998|gb|CP001923.1|	0.473	0.025	0
Acinetobacter baumannii SDF plasmid p2ABSDF	gi|169150750|emb|CU468232.1|	0.428	0.032	0.012
Acinetobacter baumannii SDF plasmid p1ABSDF	gi|169150741|emb|CU468231.1|	0.409	0	0.016
Acinetobacter baumannii str. AYE plasmid p3ABAYE	gi|169147050|emb|CU459140.1|	0.345	0.032	0.03
Acinetobacter baumannii BJAB0715 plasmid pBJAB0715	gi|522376232|gb|CP003848.1|	0.293	0.025	0.025
Shewanella sp. ANA-3 plasmid 1	gi|117614903|gb|CP000470.1|	0.251	0.002	0.003
Acinetobacter baumannii str. AYE plasmid p4ABAYE	gi|169147044|emb|CU459139.1|	0.22	0	0
Acinetobacter baumannii str. AYE plasmid p1ABAYE	gi|169147024|emb|CU459137.1|	0.213	0	0.018
Aeromonas salmonicida subsp. salmonicida A449 plasmid 5	gi|142856267|gb|CP000646.1|	0.195	0.002	0.001
Acinetobacter baumannii TCDC-AB0715 plasmid p1ABTCDC0715	gi|323519903|gb|CP002523.1|	0.172	0	0
Klebsiella pneumoniae subsp. pneumoniae Kp13 plasmid pKP13a	gi|569550058|gb|CP003996.1|	0.163	0.041	0
Acidovorax sp. JS42 plasmid pAOVO02	gi|120608609|gb|CP000541.1|	0.061	0.358	0.816
Alicycliphilus denitrificans BC plasmid pALIDE02	gi|317119756|gb|CP002451.1|	0.053	0.227	0.509
Emticicia oligotrophica DSM 17448 plasmid pEMTOL02	gi|387857508|gb|CP002963.1|	0.038	0.226	0.006
Novosphingobium sp. PP1Y Lpl large plasmid	gi|333936448|emb|FR856860.1|	0.029	0.212	0.209
Emticicia oligotrophica DSM 17448 plasmid pEMTOL04	gi|387857618|gb|CP002965.1|	0.029	0.2	0.01
Ralstonia eutropha JMP134 plasmid 1	gi|72123597|gb|CP000093.1|	0.038	0.198	0.469
Burkholderia cepacia AMMD plasmid 1	gi|115286659|gb|CP000443.1|	0.028	0.168	0.464
Acidovorax sp. JS42 plasmid pAOVO01	gi|120608524|gb|CP000540.1|	0.021	0.165	0.574
Bacillus megaterium WSH-002 plasmid WSH-002_p1	gi|345447048|gb|CP003018.1|	0.048	0.158	0.067
Cupriavidus metallidurans CH34 megaplasmid	gi|288237308|gb|CP000353.2|	0.134	0.145	0.094
Alicycliphilus denitrificans BC plasmid pALIDE01	gi|317119630|gb|CP002450.1|	0.024	0.142	0.322
Bacillus megaterium QM B1551 plasmid pBM400	gi|294351974|gb|CP001987.1|	0.074	0.141	0.097
Paracoccus aminophilus JCM 7686 plasmid pAMI2	gi|258559848|gb|GQ410978.1|	0.032	0.14	0.162
Paracoccus aminophilus JCM 7686 plasmid pAMI1	gi|529583285|gb|CP006651.1|	0.03	0.118	0.096
Achromobacter xylosoxidans A8 plasmid pA81	gi|310764373|gb|CP002288.1|	0.02	0.109	0.324
Novosphingobium aromaticivorans DSM 12444 plasmid pNL2	gi|145322317|gb|CP000677.1|	0.016	0.107	0.131
Arthrobacter aurescens TC1 plasmid TC1	gi|119951388|gb|CP000475.1|	0.036	0.102	0.197
Ralstonia pickettii 12D plasmid pRp12D02	gi|240868673|gb|CP001647.1|	0.011	0.098	0.256
Cupriavidus metallidurans CH34 plasmid pMOL28	gi|288259477|gb|CP000355.2|	0.023	0.085	0.218
Tistrella mobilis KA081020-065 plasmid pTM4	gi|388532359|gb|CP003240.1|	0.06	0.097	0.134

Figure 6 Heatmap of the top rank plasmids and their sequence abundance of three datasets of water studied

Discussion

Our results confirmed the increasing need for knowledge on measurement and control of antibiotic resistance in agriculture and aquaculture, in order to mediate the problem of excessive antibiotic usages. But how much the farms contribute to the antibiotic resistance, what factors impact most and how sound antibiotic usage should be effective, is still a matter of debate adjusted to each farm system. Prior to addressing these questions, we wanted to know how local caging fish farm got involved in antibiotic resistance and whether it spread the ARGs to the environment. At present, large-scale studies on antibiotic resistances in the environment have become possible through metagenomics as even small numbers of bacteria in soil and aqua samples can be screened for antibiotic resistances. In this study we were able to confirm the power of metagenomics in the evaluation of ARGs in aqua-biomes of upstream, downstream and the caging fish farm of Taasan-Blangpla canal, where 1057 tones of red tilapia were produced yearly (Ingthamjitr *et al.*, 2017).

Distribution of bacteria and human intestine microbes

To evaluate the bacteria strains in the fish farm, we asked the question as to whether bacteria strains in the upstream location have been spread to the fish farm, or that the occurrence of the strains was the direct effect of the farm. Our data present T1site with the highest prevalence that may be affected by the swine, cattle and poultry farm close to the sample site. The most common strains of T1 was different from T1 and T2 suggested that the majority of the bacteria at T1 disseminated less to the fish farm and downstream sites, and so did for the spreading of the fish farm downstream. It follows that the bacteria in the fish farm has their own microclimate in promoting their specific bacteria populations. There is the most prevalence strain shared only in T2 and T3 suggested an unknown differential selection. In addition, the distribution of gut microbes was congruent to that of bacteria strains which emphasized the unique ecology for site specific bacteria.

The distribution of plasmid

The increase of ARGs is determined by selective pressure by biotic and abiotic factors. In addition, we assumed that the effective horizontal transfer between and among strains achieve by plasmids, transposable elements and virus does happen, and if so as to whether they are involved in ARGs dissemination to downstream sites. Our results now revealed that the distribution of most common plasmids of T1 was specific for that area, while the plasmids in the fish farm and downstream site were more relevance, and so indicated that plasmids of fish farm got less influence from the upstream site and being common for the farm and downstream water. The most observed sequences shared between plasmids of the fish farm and downstream were repeats of transposable elements, directly pointing their putative role in sequence transfer.

The distribution of antibiotic resistance genes

Although the ARGs found in T1 and T2 were similar in their antibiotic classes, their genes were different, which indicated little or no ARG distribution between the two sites. None of the dominating resistance of T1, quinolone ARG, was found in the fish farm and downstream site, then it was more likely that no antibiotic residues from T1 remaining in the downstream sites. Also, for the ARGs of the fish farm were not in the downstream site. Therefore, it became obvious that the fish farm operation did not release antibiotic residues in the canal during the studied period.

Does the caging fish farm contribute antibiotic resistance genes in the Taasan-Bangpla canal?

Antibiotic resistance is the natural phenomenon of bacteria to fight against each other. Even in the pristine areas the ARGs are common. In the husbandry and fish farm, the ARGs are more prevalence too even though antibiotics are not used in the farm that explanations may diverse. For example, the feed contains fungi produced antibiotics, the feed materials produced from animals feed with antibiotics, the gut bacteria produced antimicrobial substances (Hong *et al.*, 2018, Muziasari *et al.*, 2017, Shah *et al.*, 2014). If the environments promote the strong selection, the ARGs will persist and transfer among bacteria community. In our study, it

showed well that both household and fish farm have more diversity and prevalence than the downstream site where being without farm activities. This prevalence may correspond to the study of Muziasari *et al.* (2017) who indicated the fish feces were the sources of ARGs and transposable elements. They also suggested that co-selection of ARGs was possible by the processes of integrases and transposases. The three sites shared bacterial strains, ARGs, repeats of transposable elements that occurred at low frequency in their site, indicating that they may be endemic of this area studied. In contrast, there were specific bacterial strains, ARGs and repeats—which were all detected with high frequency—that pointed out the site-specific selections. These specific strains, genes and repeats were prevalence in restrict regions suggesting that the farm and their site activities had not impacted the antibiotic resistance in downstream—which corresponded to the free antibiotic feed, as mentioned by the farmers we interviewed. Remarkably, the fish farm and the downstream site did not share ARGs but did share the repeated mobile elements. It was likely that these repeat sequences were involved in other selections, for example, heavy metal resistance.

Our study was done during January 2018 with the water flow rate of 36.47 x 106 m3/month which was higher than the months before. For this reason, the antibiotic residues might be flooded away from the downstream water, which might explain the low ARGs found in the downstream site. Therefore, the year-round studies are needed.

Conclusion

In this study we have shown the great power of metagenomics in evaluating soil and aqua microbiomes, and their effects of human activity in and nearby the sites of study. We have demonstrated that relative occurrences of ARGs, plasmids, transposons and bacteria shed light on their biology, distribution and selection processes. However, the complexity of their interactions requires additional projects focusing on other and well-defined cases of human – animal and microbiome interactions.

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