

BROADENING THE TARGETS FOR MICROBIAL WATER QUALITY

THE CLOSE ASSOCIATION BETWEEN BACTERIA COMMUNITY COMPOSITION AND ELEVATED NUTRIENTS IN ESTUARIES COULD BE EXPLOITED TO SELECT A RANGE OF WATER QUALITY INDICATORS

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ABSTRACT

Bacterial community composition can change as a result of increased nutrient loads and may be useful for assessing ecosystem health in estuaries.

However, the ability to understand how bacterial communities respond to increased nutrient concentrations is limited by the paucity of community-level bacterial base data, in particular for tropical estuaries. Our aim was to describe and compare the bacterial community in the water column and sediments across tropical tidal creeks in Darwin Harbour (NT).

The authors assessed the relationship between communities and increased nutrient loads, comparing sites with sewage effluent inputs to control sites. In this tropical estuary, bacterial species richness and diversity in water increased with increased nutrient load. This result suggests that there is an untapped resource of bacteria that should be explored as potential water quality indicators for receiving environments. The authors' work has shown that taxa such as *Aeromonas*, *Azomonas* and various cyanobacteria have potential as water quality indicators, not just for public health but also as measures of ecosystem health.

Keywords: Bacteria, sewage effluent, tropical macro-tidal creeks, water quality indicator.

INTRODUCTION

Darwin Harbour (DH) in northern Australia (Figure 1) is currently undergoing considerable on- and off-shore infrastructure and residential development, and receives approximately 10,650 ML yr⁻¹ of sewage effluent discharged from five treatment plants. Sewage effluent is currently considered to be the largest water quality management issue in the harbour (Fortune and Maly, 2009).

DH is a macro-tidal environment, and has been shown to be poorly flushed and to have a complex bathymetry that

can trap pollutants inshore for long periods (Williams *et al.*, 2006). Localised hotspots of poor water quality include Buffalo Creek (BC), which has the most degraded water quality in DH (DLRM, 2012), and Myrmidon Creek (MC), which both receive treated sewage effluent.

Smith *et al.* (2012) found that benthic nutrient fluxes increased and denitrification efficiency (DE) decreased in response to increased nutrient loads in BC. Consequently, the bulk of nitrogen in the sediments in BC is released back into the water column as bioavailable ammonium



Fishing at the mouth of Buffalo Creek, NT.



Figure 1. Map of study sites, Darwin, Australia. The two non-effluent reference creeks were WA = West Arm ($2^{\circ}33'35\text{S}$ $130^{\circ}47'02\text{E}$) and HC = Hudson Creek ($12^{\circ}28'31\text{S}$ $130^{\circ}56'09\text{E}$). The three effluent-impacted sites were EP = East Point ($12^{\circ}24'10\text{S}$ $130^{\circ}49'26\text{E}$), MC = Myrmidon Creek ($12^{\circ}29'57\text{S}$ $130^{\circ}56'47\text{E}$) and BC = Buffalo Creek ($12^{\circ}21'32\text{S}$ $130^{\circ}54'38\text{E}$). STP = sewage treatment ponds.

and nitrates which, combined with the sewage, contributes to increased algal biomass and degraded water quality. Conversely, the effect of the sewage load in MC is confined to the water column and appears to be temporary and highly localised (Smith *et al.*, 2012).

Worldwide there is a call for broadening microbial water quality assessment to use a range of ecosystem and potential pathogen indicators. Faecal coliform bacteria were initially used as indicator organisms for water because it was thought that they were primarily associated with the faeces of warm-blooded animals in natural waters (APHA, 1995). However, they and enterococci are also associated with the faeces of 'cold-blooded' animals (Harwood *et al.*, 1999). It is now well understood that *Escherichia coli*

and enterococci can survive and be detected in a variety of non-faecal sources (Byappanahalli *et al.*, 2012). Their capacity to survive in the absence of a host makes it difficult to accurately monitor human faecal pollution in the receiving environment (Wang *et al.*, 2013; Lee and Silk, 2013), particularly if they have little bearing on pathogen survival and retention.

The call for broadening the targets for water quality assessment has also been driven by the application of next-generation sequencing (NGS) to environmental samples, which has allowed significant advances in our understanding of bacterial community dynamics. NGS has created an unprecedented inventory of microbial communities associated with faecal sources, allowing re-examination of which taxonomic

groups are best suited as informative indicators (McLellan and Eren, 2014). This includes advances in microbial source tracking (MST) to discriminate between human and non-human sources of faecal contamination in the environment (Ahmed *et al.*, 2007; Gourmelon *et al.*, 2007; Field *et al.*, 2003; Harwood *et al.*, 2003; Noble *et al.*, 2003; Stoeckel *et al.*, 2004; Neave *et al.*, 2014; Malham *et al.*, 2014). NGS has allowed us to re-focus and examine the entire microbial community rather than making a *priori* decisions about what marker to choose, thus it provides an opportunity to assess more potential faecal and pathogen indicators.

While these new approaches have opened the door to ecological scale microbial assessment and have great potential to resolve microbial community dynamics (Stewart *et al.*, 2008), we still have much to learn about how microbial communities respond to disturbance. At a more fundamental level, there are many regions and ecosystems for which we have no community level microbial data from which we can pose informed questions about ecosystem health and processes. DH is one such region, so the aim of this study was to compare the bacterial community composition in sewage effluent impacted, and control creeks with no point source sewage effluents. From this baseline knowledge the authors aimed to increase their understanding of what makes the bacterial communities different, if in fact they are, what the implications might be for monitoring, and how potential indicators here compare to those from better studied temperate regions.

METHODS

Sites And Physicochemistry

Darwin Harbour is a macrotidal estuary influenced by a tropical monsoonal climate and a highly seasonal rainfall pattern. Buffalo Creek (BC) in Shoal Bay has a narrow, meandering channel that flows through mangroves, with small intertidal mudflats on the meander bends and a large sand bar across the mouth, which inhibits tidal exchange. At the upstream end of the creek BC receives secondary-treated

Table 1. Nutrients in filtered water and grain size in sediment grabs at each study site.

Site	ON μM	Total N μM	NO ₂ -N μM	NO ₃ -N μM	Total P μM	PO ₄ -P μM	TSS mg/L	VSS mg/L	<63μm %
BC	17.2	41.5	6.20	5.30	36.2	9.80	50	23	50.5
MC	5.5	5.8	0.04	0.03	7.8	0.80	52	11	100
EP	10.7	11.0	<0.02	0.03	7.4	4.50	57	16	0
HC	1.3	2.3	<0.02	<0.02	0.7	0.03	46	8	100
WA	2.0	2.1	<0.02	0.02	0.3	0.05	34	6	90.3

sewage effluent (annually TN 79t, TP 43t, NH₃ 12t; flow 6,128 ML, (PWC 2006)) from a sewage treatment plant (STP) (Figure 1).

BC is hypereutrophic with elevated benthic nutrient fluxes and denitrification depressed, with denitrification efficiency (DE) at <10% (Smith *et al.*, 2012). Myrmidon Creek (MC) has a wide and relatively straight channel surrounded by mangroves with extensive mudflats at low tide. MC receives secondary-treated sewage effluent (annually TN 69t, TP 18t, NH₃ 40t; flow 2,558 ML, (PWC 2006)) from a STP, which is discharged into mangroves halfway along the creek. Unlike BC, the impacts from the increased nutrient loads in MC are confined to the water column and are temporary and highly localised, and DE is approximately 90% (Smith *et al.*, 2012).

East Point (EP) is a rocky, coastal point located at the entrance to DH and sewage subjected to chemically-enhanced sedimentation with high median levels of ammonia is discharged (annually TN 122t, TP 28t, NH₃ 88t; flow 4,752 ML, (PWC 2006)) offshore into relatively open sea. Hudson Creek (HC) is located in a catchment with light industrial land use with no known sewage discharges, and West Arm (WA) channel is an extensive mangrove habitat with intertidal mudflats and no known sewage discharges.

Duplicate sediment and water samples were collected from each of the five sites (Figure 1) on incoming tides in October and November 2009, to measure grain size, water quality (total suspended solids (TSS), nutrients and chlorophyll) and the bacterial community structure. Sediment grab samples were

separated into two grain-size fractions (mud: <63 μm and sand: >63 μm) using a 63μm sieve. Each fraction was oven-dried at 60°C and weighed, and the relative percentages of each fraction calculated. Total suspended solids (TSS) in unfiltered water samples and volatile suspended solids (VSS) (to approximate the amount of organic matter present in the solid fraction of each water sample) were measured (Water Chemistry Laboratory, Department of Resources, Northern Territory Government) using standard methods (APHA, 1998). Total Kjeldahl nitrogen (TKN) (a measure of organically bound nitrogen and ammonia), total nitrogen (N), total phosphorus (P), nitrite (NO₂), nitrate (NO₃), phosphate (PO₄-P), ammonia (NH₃), chlorophyll-a were measured in filtered water samples (Northern Territory Environmental Laboratories, Darwin) using standard methods (APHA, 1998).

Bacteria Analysis

DNA was extracted from 200–800 mL water per sample using the MoBio Power Water or Power Soil DNA isolation kits (Geneworks, SA, Australia) according to the manufacturer's instructions. A section of a bacterial gene present in all bacteria (16S rRNA V6-region) was amplified (Sogin *et al.*, 2006) and these products were sequenced (Roche GS FLX 454) at the Australian Genome Research Facility (AGRF) in Brisbane, Queensland, Australia. The sequences were curated (Maidak *et al.*, 1997; Schloss *et al.*, 2009) and clustered into operational taxonomic units (OTUs).

These OTUs were used to represent the bacterial community in subsequent analyses. The OTUs were compared against databases to assign bacterial identities (the SILVA reference databases and Mothur taxonomy files).

The top 25 most abundant OTUs in the Mothur shared file was converted to a Cytoscape network file, and a network was constructed containing both OTUs and sites as nodes, and edges were drawn between OTUs and the site in which they were detected. The weight of the edge was proportional to the abundance of the OTU. The networks were visualised using Cytoscape v2.8.3 (Smoot *et al.*, 2011). OTUs were used to measure the diversity (Shannon index (*H*) and Simpson's reciprocal index (*1-D*)) and richness (*Chao* index) of bacteria at each site. The authors also measured evenness (*E*), i.e. whether the community was dominated by particular OTUs or evenly spread. The abundances for each sample were relative abundances obtained by dividing the number of sequences for the OTU by the total number of sequences.

Statistical Analysis

Data were analysed using PRIMER 6 (Plymouth Routines in Multivariate Ecological Research, version 6) (Clarke and Warwick, 2001). Highly collinear water quality variables were excluded (*R* > 0.95), retaining one variable from the group, log transformed and normalised. An unconstrained principal coordinates ordination (PCO) was applied to examine the differences in water quality data between sites. The bacterial assemblages were analysed separately for water and sediment samples. A Bray-Curtis similarity matrix was computed on averaged, standardised, square-root transformed sequence data so that samples with different sequence counts could be compared, and the influence of highly abundant OTUs was reduced. A distance-based linear regression model (DistLM) was applied, choosing the model with the lowest BIC (Bayesian information criterion) to

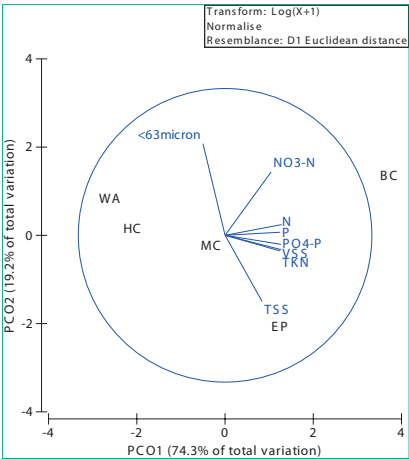


Figure 2. Principal coordinates ordination (PCO) of water nutrient variables and sediment size composition at each sample site. Vectors show the strength and direction of partial correlations of the co-variables with the PCO axes and include: <63micron = grain-size fraction (mud: <63 µm and sand: >63 µm), NO₃-N = nitrate, N = total nitrogen, P = total phosphorus, PO₄-P = phosphate, TKN = total Kjeldahl nitrogen, VSS = volatile suspended solids and TSS = total suspended solids. Vectors for VSS and TKN overlap.

analyse which water quality variables best explained the variation in OTU composition between sites. Models were visualised using constrained distance-based Redundancy Analysis (dbRDA). Vectors shown (Figure 3) are partial correlations of predictor variables with the ordination axes. Relationships between bacterial community composition and water quality variables were also tested using the non-parametric permutational RELATE procedure (Clarke and Warwick, 2001). A PCO was also applied to examine the differences between bacterial communities (OTU data) in water between replicates within sites.

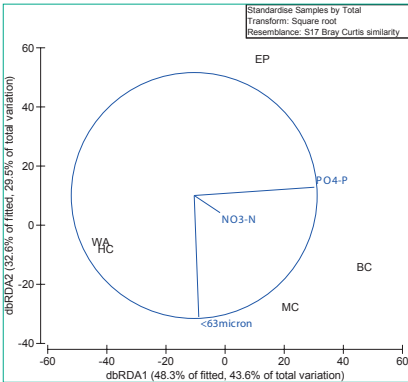


Figure 3. A dbRDA plot of bacteria (operational taxonomic unit or OTU) community diversity in water at each sample site. The vectors <63micron, NO₃-N and PO₄-P show the strength, i.e., length and direction of the relationship between these parameters, which best explained the microbial community in the distance-based linear model and the dbRDA ordination axes. See Figure 2 for co-variate abbreviations.

Data Availability

All V6 sequence data are available in the NCBI Sequence Read Archive under the BioSample accession numbers SAMN02359762 to SAMN02360092.

RESULTS AND DISCUSSION

Sites And Physicochemistry

MC and HC sediments were all mud, WA was mostly mud, BC had mixed sediment with equal parts mud and sand, and EP was all sand (Table 1). TSS was highest in the sewage-input sites and slightly lower elsewhere. Organic matter present in the solids fraction (VSS), showed similar trends and was highest in BC and lowest at WA. Nutrient concentrations were highest in BC, and EP was higher than MC (Table 1). At MC and EP, most of the nitrogen was organically

bound, but at BC there was a greater contribution from NO_x. The degree of impact consistent with sewage effluent at each site can be defined as (highest to lowest): BC > EP > MC > HC = WA.

Water quality varied between sites. BC's position in the principal coordinates ordination (PCO) plot (Figure 2) was associated with a combination of high NO_x, increased TKN, PO₄-P, N and P. In contrast, EP was associated with high TSS. The no sewage-input sites (WA) and industrial site (HC) were similar and their position was consistent with the lowest nutrient concentrations and a high clay/silt content (<63µm).

Bacteria Community Composition And Diversity

The bacteria communities were represented by sequences or OTUs, and the average number of OTUs obtained per sample after processing was 4,085. There were three clusters of sites with similar bacterial compositions (Figure 3). One cluster consisted of the reference creeks with very similar bacterial compositions, one of MC and BC and a third one of EP. Distance-based multivariate linear regression modelling was used to relate variations in the bacterial community in water at different sites to water quality parameters, i.e. water nutrient levels as well as sediment size. This model related high PO₄-P and NO_x to the similar bacterial communities of MC and BC, while the bacterial composition of MC, HC and WA was related to the <63µm (clay/silt) levels in sediment (Figure 3). There was a significant correlation between bacterial community composition and nutrient concentration (Spearman's rho = 0.66; P = 0.013).

Table 2. Ecological indices for bacteria in water at each sample site. Indices were the observed richness (the number of OTUs), the Shannon-Wiener index (H) and Simpson's reciprocal index (1-D) measures of diversity, the index of evenness (E) and the Chao non-parametric estimator of species richness.					
Site	S _{obs} (S)	Chao	Shannon (H)	Simpson's reciprocal index (1-D)	E = H/lnS
BC	677	1505	5.3	60.1	0.8
MC	650	1477	4.8	28.5	0.7
EP	632	1553	4.6	16.3	0.7
HC	382	1112	3.5	6.5	0.6
WA	310	767	3.2	5.9	0.6

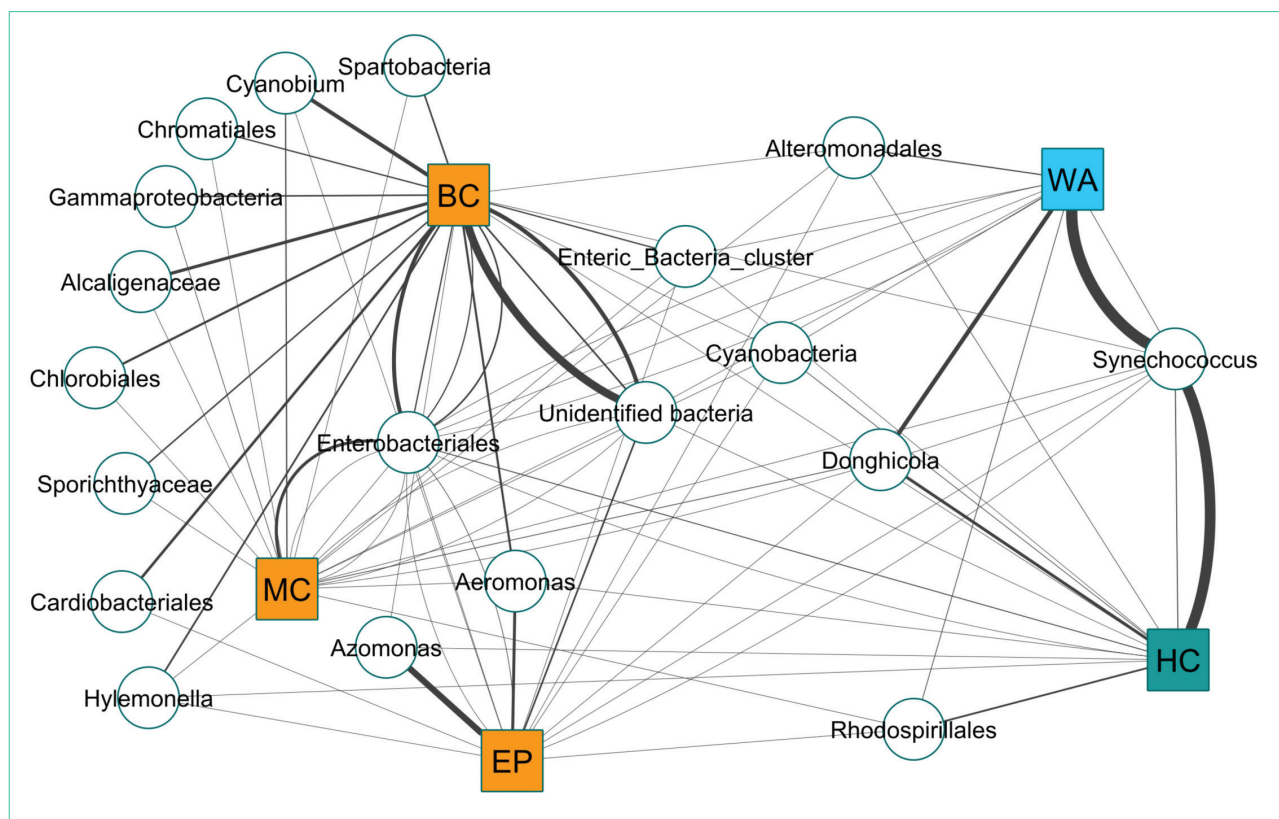


Figure 4. A cytoscape network visualisation of the top 25 most abundant OTUs (at 97% similarity). The network contained both OTUs and sites as nodes, and edges were drawn between OTUs and the site in which they were detected. The weight of the edge was proportional to the abundance of the OTU.

Bacterial community composition (the OTUs) was compared between sites using diversity and related indices (Table 2). At the most impacted site, BC, the bacteria community in water had more species (*S* and *Chao*), was more diverse (*H* and *1-D*) and relatively evenly distributed. Species richness, diversity and evenness was highest at BC and in general followed the same trends for the level of impact as defined from the water quality data, i.e., BC > MC ≥ EP > HC = WA.

Bacteria That Distinguish Sewage-Impact From Non-Sewage Impact Sites

The top 25 OTUs that contributed most to the dissimilarity between the bacterial communities from the effluent-impacted sites (BC, MC and EP) and control sites (HC and WA) were visualised using Cytoscape (Figure 4). In this figure the thickness of the lines is directly proportional to the average standardised abundance of this taxa in this group of samples. BC had a number of OTUs that were unique or only occurred at

very low levels at the other sites, including those belonging to the Enterobacteriales, *Alcaligenaceae*, *Chlorobiales*, *Hylemonella* and enteric bacteria. There were some bacteria that were common to only these MC and BC. These included a *Cyanobium*, a *Spartobacteria* and a cyanobacterium. EP was dominated by an ammonia-utilising *Azomonas* and the potential human pathogen *Aeromonas*, which was also present but not dominant at BC. The cyanobacteria *Synechococcus*, *Donghicola*, purple non-sulfur bacteria *Rhodospirillales* and an *Alteromonadales* were more prevalent in the reference creeks WA and HC compared to the impact creeks.

Implications For Monitoring

In this tropical tidal estuary, the authors showed that bacterial community-level changes were associated with nutrient load. In water, the microbial community was more diverse at the site with the highest level of nutrients (BC) and diversity decreased with decreasing

levels of nutrients. This alignment between nutrients and bacteria in water bodies well for biological monitoring in this system, particularly if bacteria signatures specific to the most impacted creek (BC) can be identified in more extensive temporal and spatial studies.

Some studies of bacteria communities have not shown a change in response to wastewater (Köchling *et al.*, 2011), but many more support the authors' findings (Sipura *et al.*, 2005; Ford *et al.*, 2004; Castine *et al.*, 2009; Jorgensen *et al.*, 2012 and references therein). The approach the authors used (16S rRNA pyrotag sequencing) is gaining wider acceptance and can provide robust and reliable abundance and community structure data within natural microbial communities (Pilloni *et al.*, 2012).

In the study, the bacteria identities were typical of those reported for water and sediment in coastal and mangrove environments, including studies associated with elevated nutrients (Gomes *et al.*, 2008; Köchling

et al., 2011). At BC the community was remarkably even and no single OTU was dominant. *Aeromonas*, which was abundant at EP and present at BC, is readily detected in sewage ponds (McLellan *et al.*, 2010) and sewage-associated receiving environments (VandeWalle *et al.*, 2012; Khan *et al.*, 2009). EP was dominated by *Azomonas*, which can utilise ammonia and was not detected at any other site. This difference between BC and EP may reflect the different treatments, because at EP the discharge is not retained in ponds but is discharged directly from a chemically assisted treatment plant and has high median levels of ammonia.

The Enterobacteriales were interesting in our data. This family contains taxa used in conventional water quality assessment, including *Salmonella*, *Escherichia coli*, *Enterobacter* and *Klebsiella*. They also include representatives of human faecal microbiota isolated from human faeces (Newton *et al.*, 2015). Some members of this order, however, also include environmental bacteria and, indeed, in our study two Enterobacteriales OTUs were detected at all sites. Members of the Enterobacteriales were abundant at BC, but not at MC or EP.

The lack of resolution beyond family highlights one of the issues with NGS. Taxonomic resolution is limited because the sequence reads are short (approximately 300bp) so our view is that, while this high throughput approach provides an opportunity to measure bacterial community diversity across time and space at a level that has not been previously possible, it is essentially a first step for identifying indicators for different purposes: public health vs ecosystem health. For example, the approach provides a capacity to search for indicators of ecosystem function change – e.g. *Azomonas*, and pathogens e.g. *Aeromonas*, but species or strain-specific assays using quantitative PCR, for example, would need to be developed as a follow-up step for routine screening, particularly where no sufficiently specific qPCR test currently exists, or where more

extensive wildlife faecal testing shows they are not human specific. NGS databases, however, are rich sources of information for follow-up studies, so they are a valuable resource. For example, the data from this study contain a number of potential pathogens that occur naturally, such as *Vibrios*, and these data can be mined at a later date to address the important issue of effluent and naturally occurring bacteria in seawater.

CONCLUSION

The authors compared bacterial community composition between sites that ranged from significant inputs of sewage effluent through to no known sewage effluent inputs. Increased nutrient loads were associated with changes in the bacterial community composition and, interestingly, even within sewage-input sites there were differences between the bacterial communities, suggesting other factors also influence the bacterial community composition. Water-derived bacteria had increased diversity and richness at the most impacted sites and taxa such as *Aeromonas* and *Azomonas*, and various cyanobacteria, show promise as indicators. So too did members of the Enterobacteriales, but resolution was only to family, so further tests would need to be developed for routine testing. Although taxonomic resolution using NGS is still limited if searching for species level indicators, the high throughput and robust nature of the approach lends itself to ecologically relevant temporal and spatial impact studies.

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