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## Environmental DNA highlights the influence of salinity and agricultural run-off on coastal fish assemblages in the Great Barrier Reef region<sup>☆</sup>

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## ABSTRACT

Agricultural run-off in Australia's Mackay-Whitsunday region is a major source of nutrient and pesticide pollution to coastal and inshore ecosystems of the Great Barrier Reef. While the effects of run-off are well documented for the region's coral and seagrass habitats, the ecological impacts on estuaries, the direct recipients of run-off, are less known. This is particularly true for fish communities, which are shaped by the physico-chemical properties of coastal waterways that vary greatly in tropical regions. To address this knowledge gap, we used environmental DNA (eDNA) metabarcoding to examine fish assemblages at four locations (three estuaries and a harbour) subjected to varying levels of agricultural run-off during a wet and dry season. Pesticide and nutrient concentrations were markedly elevated during the sampled wet season with the influx of freshwater and agricultural run-off. Fish taxa richness significantly decreased in all three estuaries ( $F = 164.73$ ,  $P < 0.001$ ), along with pronounced changes in community composition ( $F = 46.68$ ,  $P = 0.001$ ) associated with environmental variables (largely salinity: 27.48% contribution to total variance). In contrast, the nearby Mackay Harbour exhibited a far more stable community structure, with no marked changes in fish assemblages observed between the sampled seasons. Among the four sampled locations, variation in fish community composition was more pronounced within the wet season ( $F = 2.5$ ,  $P = 0.001$ ). Notably, variation in the wet season was significantly correlated with agricultural contaminants (phosphorus: 6.25%, pesticides: 5.22%) alongside environmental variables (salinity: 5.61%, DOC: 5.57%). Historically contaminated and relatively unimpacted estuaries each demonstrated distinct fish communities, reflecting their associated catchment use. Our findings emphasise that while seasonal effects play a key role in shaping the community structure of fish in this region, agricultural contaminants are also important contributors in estuarine systems.

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## 1. Introduction

In agricultural landscapes, elevated loadings of nutrients, pesticides and suspended material pose a significant threat to estuarine and coastal biodiversity (Stauber et al., 2016; Mondal et al., 2018; Covert et al., 2020; Montagner et al., 2022). Many marine vertebrates are reliant on estuaries during different life cycle stages, e.g., feeding, migration, shelter, breeding and nursery grounds (Potter et al., 2013; Harasti et al., 2017; Schilling et al., 2018). In north-eastern Australia, estuary-dependent species (e.g. barramundi *Lates calcarifer* and mangrove jack *Lutjanus argentimaculatus*) are pivotal to the region's economic and social values through their contributions to recreational and commercial fisheries (Halliday & Robins, 2001; Skirtun et al., 2012; Taylor et al., 2012). Given the growing intensity and diversity of human activities in coastal catchments, there is an urgent need to better understand how agricultural run-off impacts these habitats and the fish communities they support (Donázar-Aramendía et al., 2019).

Northern Australia has a tropical climate, and the physico-chemical attributes of estuaries are highly influenced by distinct rainfall patterns during wet and dry seasons (Halliday & Robins, 2001). High rainfall during the wet season (November–April) not only causes a pronounced reduction in salinity within estuaries but is also the major vector for the transport of terrestrially derived contaminants via freshwater plumes (Brodie et al., 2010; Kroon et al., 2012; Huggins et al., 2017). These freshwater run-off inputs are the primary source of pollution into the Great Barrier Reef (GBR) region, impacting critical fish habitats that include seagrass meadows, soft-sediment infauna, hard substrate communities and corals (Haynes et al., 2000; Queensland Government, 2009; De'ath and Fabricius, 2010).

Pesticides are widely used in Australia with concentrations of their active ingredients in monitored waterways frequently exceeding national water quality guideline values (Brodie & Landos, 2019; Warne et al., 2022). Warne et al. (2020) examined over 2000 samples from 15 waterways draining into the GBR lagoon and found quantifiable pesticide mixtures in 80% of the samples. Atrazine (and its metabolites), diuron, imidacloprid, hexazinone, and 2,4-dichlorophenoxyacetic acid (2,4-D) were among the most frequently detected pesticides (Warne et al., 2020). In highly contaminated estuaries within the GBR region, elevated pesticide concentrations observed during the wet season have been correlated with alterations in the hepatic transcriptomes of barramundi, potentially affecting lipid metabolism and the capacity to store energy (Hook et al., 2018). Pesticides in estuaries associated with growing sugarcane have also been shown to significantly correlate with liver vitellogenin transcription levels in barramundi, and to a lesser degree, coral trout (*Plectropomus leopardus* and *P. maculatus*) (Kroon et al., 2015). Herbicides such as atrazine and diuron directly impact diatoms, periphyton, phytoplankton and macrophytes, and have indirect effects on non-targeted taxa such as fish, through habitat and resource quality degradation and the disruption of trophic networks (Dewey, 1986; Denoyelles et al., 1989; DeLorenzo et al., 2013; Miranda, 2018; Wood et al., 2019). However, quantifying the impacts of pesticides on fish communities remains challenging, as their adverse effects need to be evaluated in the presence of other co-occurring anthropogenic and natural stressors (Scholz et al., 2012; Thomas et al., 2020; King et al., 2022a; King et al., 2022b). Monitoring multiple stressors and seasonal variability together with fish communities is essential for assessing ecosystem health and understanding how fish assemblages respond to contaminant-enriched run-off and seasonal changes in freshwater inflows (Robins et al., 2005; Mayer-Pinto et al., 2015; Baird et al., 2016).

Conventional approaches for monitoring fishes in coastal environments include netting, trawling, acoustics, underwater visual censuses (UVC), and the use of remote and driver-operated vehicles (Harmelin-Vivien & Francour, 1992; Murphy & Jenkins, 2010; Baker et al., 2016; Raoult et al., 2020). These approaches can be time-consuming, expensive, invasive, labour intensive, and dependent upon expertise

and equipment (Eustice et al., 2004; Murray et al., 2006). In the Mackay-Whitsunday region, routine monitoring of freshwater fish is performed via electrofishing, however, this approach is not applicable to estuarine environments due to the conductivity of estuarine waters (Mackay-Whitsunday-Isaac Healthy Rivers to Reef Partnership, 2022). Environmental DNA (eDNA) metabarcoding is a genetic tool that is reshaping the way aquatic (and terrestrial) systems are being surveyed, providing a comprehensive overview of the resident taxa by using remnant genetic material in water, sediment or other abiotic matrices, which can complement or in some cases replace conventional methods of monitoring (Andruszkiewicz et al., 2017; Boussarie et al., 2018; Ruan et al., 2022; Xiong et al., 2022; Rey et al., 2023). When employed in conjunction with the measurement of environmental and chemical variables, eDNA metabarcoding can provide a depth of information on fish habitat use and stressors, e.g. Xie et al. (2021).

This study examined the relationships and interplay between natural (e.g., salinity, turbidity, chlorophyll *a*) and anthropogenic (i.e., chemical signatures of agricultural run-off) stressors on estuarine fish communities within the Mackay-Whitsunday region of the Great Barrier Reef. Fish biodiversity data was obtained via eDNA metabarcoding of two fish-specific mitochondrial 12S rDNA markers from replicated sites within four locations (3 estuaries and one harbour) subjected to various degrees of agriculture-induced stressors during a dry and a wet season. We predicted that fish richness would decline and community composition would shift from the dry to the wet season due to a reduction in salinity and higher loadings of pesticides and nutrients. Furthermore, we hypothesised that fish community composition would be altered by seasonal and location differences in agricultural inputs, most notably pesticide and nutrient concentrations.

## 2. Methods

### 2.1. Study area

The Mackay-Whitsunday region typifies the marked differences in seasonal precipitation associated with tropical regions. Annual rainfall is around 1540 mm, with 75% of this typically occurring in the wet season (Bureau of Meteorology & CSIRO, 2019). Mean local rainfall in the region ranges between 24.2 and 59.9 mm during the dry season (May–October) and 95.2–326 mm during the wet season (November–April) (Bureau of Meteorology, 2022). High intensity sugarcane farming in this region causes a suite of pesticides to be delivered to the GBR lagoon in elevated concentrations, along with increased nutrient discharge (nitrogen and phosphorus) (Furnas, 2003; McKergow et al., 2005; Brodie & Landos, 2019). Sugarcane harvesting takes place prior to the wet season, from mid-August to late October, sometimes extending to December (Queensland Government, 2018). Application of pesticides and fertilisers typically occur post-harvesting, and high rainfall in the wet season induces runoff which transport nutrients and pesticides to marine waters via freshwater plumes (Brodie et al., 2010; Cook et al., 2011; Kroon et al., 2012; Queensland Government, 2018).

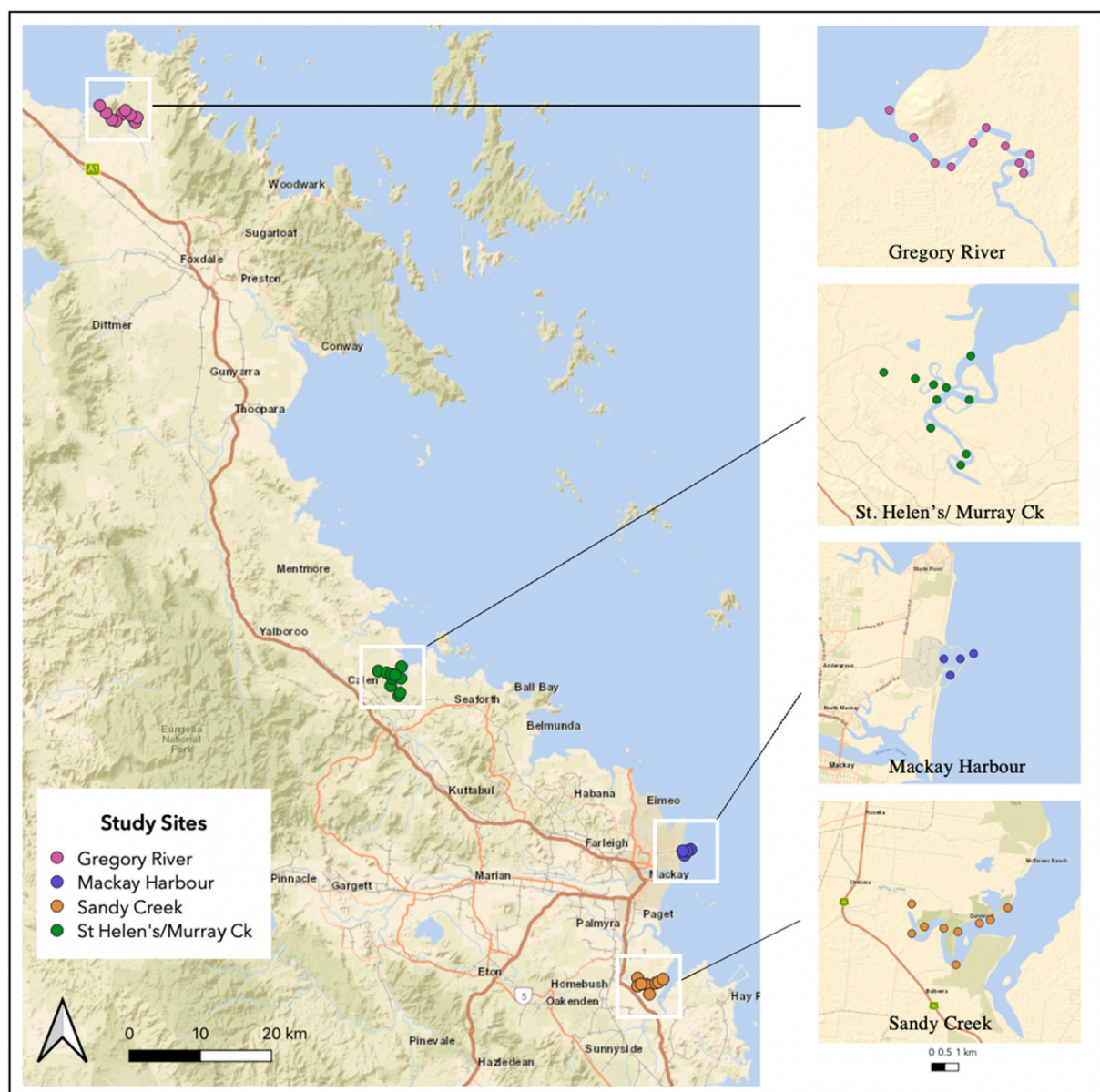
Sampling was performed at four coastal locations: three estuaries (Gregory River, St. Helen's/Murray Creek and Sandy Creek) and the Mackay Harbour which is situated at the mouth of the Pioneer River estuary (Fig. 1). These waterways were chosen to capture differences in adjacent land-uses including varying levels of agricultural run-off (pesticides and nutrients), along with industrial use in the case of Mackay Harbour. The Gregory River, the most northern location, was considered to be one of the least agriculturally impacted estuaries in the region (Newham et al., 2017; Healthy Rivers to Reef Partnership, 2021). The St. Helen's/Murray Creek estuary (henceforth referred to as St. Helen's) was categorised to be in a moderately healthy state, with nutrient concentrations (most notably nitrogen) occasionally exceeding national guidelines (Healthy Rivers to Reef Partnership, 2021). Sandy Creek was categorised as the most impacted, having the highest pesticide concentrations of any monitored catchment that drains into the Great Barrier

Reef (Smith et al., 2012; Wallace et al., 2016b). Mackay Harbour (henceforth referred to as the Harbour), typified a highly modified harbour environment (Mayer-Pinto et al., 2015). Whilst no chemical monitoring data was available for the Harbour prior to sampling, given its use and location, it is likely to be the repository of a mixture of agricultural, industrial and urban contaminants (Owen & Sandhu, 2000; Bulleri & Chapman, 2010), whilst being less influenced by seasonal changes in salinity as it is not located within an estuary.

## 2.2. Sample collection

Water samples for the analyses of eDNA, pesticides, nutrients and environmental variables were collected from 34 sites across the four study locations. This included 10 sites per estuary and four sites in the Harbour (due to its size), with two sampling events at each (Supplementary Material Table S1). The first sampling occurred towards the end of the 2018 dry season (late November 2018). Despite the lateness in the season, sampling took place during atypically dry and hot conditions for

the time of year, with the total rainfall at Mackay in November 2018 being 7.4–15.8 mm (Bureau of Meteorology, 2018). The second sampling occurred during the 2020 wet season (late January to early February 2020), with total monthly rainfall at Mackay in January 2020 being 241.6–275.2 mm (Bureau of Meteorology, 2020). Each of the three estuaries sites were sampled at approximately 1 km intervals commencing at the mouth and proceeding upstream, while in the harbour, four sites were spread out to cover locations within as well as a few hundred metres away (Fig. 1). At each site, three x 2 L surface water samples were collected in sterile Whirl-Pak bags (Nasco, America), placed on ice for transportation back to the field laboratory and kept refrigerated until filtering. Samples were filtered within 24-h of collection using 0.45 µm cellulose nitrate membranes (Advantec, Australia) and a peristaltic pump (Sentino microbiology pump, Pall Life Sciences, MI). During the 2018 dry season, up to 1 L of water per sample was filtered between two filter papers (i.e., 3 L per site). However, during the 2020 wet season, due to the high turbidity (max = 393 FNU) and associated clogging of the filters, only ~450–500 mL of water was



**Fig. 1.** Map of the Mackay-Whitsunday region (Queensland, Australia). Study locations and sampling sites for each location inset. GPS coordinates available in Supplementary Material Table S1.

filtered per sample (using three different filter papers for a maximum of 20 min each). In a few 2020 samples, it was possible to additionally filter the full 1 L of water, and the potential bias associated with differences in filtered samples is discussed later (see [Supplementary Material Fig. S1](#)). All sampling containers and apparatus were bleached (0.5 % solution) and rinsed with deionized water prior to filtering and between samples from each site to avoid cross-contamination. Following filtration, the filter papers along with any sediment residue were placed into sterilised 2 mL tubes (Eppendorf SE) and frozen in liquid nitrogen for later analysis of eDNA. A minimum of two blank samples (with deionized water) were filtered per location as extraction controls.

### 2.3. Physico-chemical, nutrient and pesticide analyses

The physico-chemical properties of the overlying waters were measured using a calibrated YSI EXO V2 multiparameter sonde (YSI, Yellow Springs, Ohio; [Supplementary Workbooks 1 and 2](#)). Water samples were also collected at each site for nutrient and pesticide analyses following Queensland Government protocols ([Department of Environment and Science, 2018](#)). Samples were collected at different depths to establish a profile along the water column and measurements were averaged for each location. All samples for nutrient and pesticide analyses were placed on ice in the field and immediately refrigerated (pesticides) or frozen (nutrients) upon returning to the field laboratory until analysis. Nutrient analyses were carried out by the Chemistry Centre of the Department of Environment and Science, Queensland Government (Queensland, Australia), a NATA (National Association of Testing Authorities) accredited laboratory. Chlorophyll *a* concentrations were determined by spectrophotometric procedures defined in method 10200 H ([APHA & AWWA, 2017](#)). Dissolved organic carbon was measured using an automated carbon analyser (wet oxidation or combustion at 680 °C over a platinum catalyst) in accordance with method 5310 D ([APHA & AWWA, 2017](#)). Total Kjeldahl values for nitrogen and phosphorus were measured via catalysed acidic block digestion with colorimetric segmented flow analyser finish according to methods 4500-N<sub>org</sub> and 4500-P B ([APHA & AWWA, 2017](#)). Targeted pesticide analyses were conducted by the Queensland Government Forensic and Scientific Services (Queensland, Australia) and a NATA accredited laboratory through direct injection using Liquid Chromatography with tandem mass spectrometry (LC-MS-MS). Water samples were filtered through a 0.2 µm membrane prior to LC-MS-MS, and the concentrations measured were of soluble pesticides (see [Supplementary Material Table S2](#) for limits of reporting).

### 2.4. DNA extraction, amplification and sequencing

DNA extractions were performed at Macquarie University's Environmental DNA and Biomonitoring Laboratory using Qiagen PowerWater kits (Qiagen, Netherlands). Prior to extraction, filter papers from a single water sample were cut into small pieces using sterile tools and placed together in a tube. Extractions were carried out following the manufacturer's instructions, except samples were vortexed for 10 min instead of 5 min and tubes were rotated every 2.5 min to ensure adequate mixing. The DNA extracts were stored at -20 °C. Two primer sets were used to target the 12S mitochondrial RNA genes in fishes – the Tele01\_12S\_F (5'-ACACCGCCGTCCTACTCT-3') and Tele01\_12S\_R (5'-CTTCCGGTACACTTACCATG-3') primers (45–96 bp) for teleost fishes, a subclass of bony fish which includes 96% of extant fish species ([Valentini et al., 2016](#)); and the Elas02\_F (5'-GTTGGTHAATCTCTGCCAGC-3') and Elas02\_R (5'-CATAGTAGGGTATCTAATCCTAGTTG-3') primers (170–185 bp) for elasmobranchs, i.e., sharks, skates and rays ([Taberlet et al., 2018](#)). Each PCR was multiplexed at the 5' ends of both forward and reverse primers using a unique 8-nucleotide label which differed by at least 5 nucleotides from each other ([Valentini et al., 2016](#); [Taberlet et al., 2018](#)).

Tele01 and Elas02 samples ( $n = 102$ ) were all amplified in triplicate.

The PCRs were performed with a total volume of 15 µL for each reaction, including, for Tele01 samples: 1x of AmpliTaq Gold™ 360 MasterMix (Thermo Fisher Scientific, Waltham, MA, USA), 0.4 µM of each primer, and 2 µL of the extracted DNA sample/control; and for Elas02 samples: 1x of AmpliTaq Gold™ 360 MasterMix, 10 µM of each primer, and 2 µL of the extracted DNA sample/control. PCRs were performed on a Mastercycler X50 (Eppendorf, Germany) under the following PCR conditions for Tele01: initial denaturation at 95 °C for 10 min; 50 PCR cycles of denaturation for 30 s at 95 °C; hybridisation at 55 °C for 30 s and elongation at 72 °C for 45 s; followed by a final elongation at 72 °C for 10 min. The PCR conditions for Elas02 were: initial denaturation at 95 °C for 10 min; 55 PCR cycles of denaturation for 30 s at 95 °C; hybridisation at 59 °C for 30 s and elongation at 72 °C for 1 min; plus, a final elongation at 72 °C for 7 min. In each plate, 4–6 field, extraction and PCR negative controls each were included. Positive controls were also included for each primer – Atlantic salmon (*Salmo salar*) and Nile perch (*Lates niloticus*) for Tele01, and gummy shark (*Mustelus antarcticus*) for Elas02. Sequencing was conducted by Ramaciotti Centre for Genomics (University of New South Wales, Australia) and performed on an Illumina MiSeq Genome Sequencer (2 × 150 bp paired-end) ([Ravi et al., 2018](#)). Bioinformatics processing of the raw 12S mtDNA metabarcoding data for Tele01 and Elas02 was carried out using the Greenfield Hybrid Analysis Pipeline (GHAP) ([Greenfield, 2017](#)), and the high-quality zero-radius sequences (zOTUs) generated were matched to the MitoFish database ([Iwasaki et al., 2013](#)) (see [Supplementary Material](#) for details).

### 2.5. Statistical analyses

All statistical analyses were performed in R version 4.1.0 ([R Core Team, 2021](#)). R code and all chemical and physico-chemical/environmental measurements are provided in the [Supplementary Material](#). In addition to pesticide data, the following environmental parameters were considered in the subsequent analyses: chlorophyll *a* (mg/L), salinity (PSU: practical salinity units), turbidity (FNU), total Kjeldahl nitrogen (mg/L), total Kjeldahl phosphorus (mg/L), and dissolved organic carbon (mg/L). The measured values of nutrients and pesticides were transformed as  $\log(x+1)$  and normalised. Prior to analyses, pesticide data during the wet season were plotted to visualise normality, and strongly correlated variables ( $r^2 > 0.95$ ) were removed from subsequent analyses (see [Supplementary Material Table S5](#)) ([Clarke & Ainsworth, 1993](#)).

Tele01 and Elas02 bioinformatics results were combined into a single dataset for analyses. Taxonomic richness was calculated for each sample as a sum of the zOTUs present per sample. A two-way ANOVA was performed to identify differences in taxonomic richness among locations, seasons, and their interaction. Any significant results (i.e.,  $P < 0.05$ ) were followed by a *post hoc* pairwise analysis. Community data (presence-absence) were examined via non-metric multidimensional scaling (nMDS) derived from the Jaccard index using the *vegdist* and *metaMDS* functions in the R package *vegan* ([Wickham, 2016](#); [Oksanen et al., 2020](#)). A Permutational Multivariate Analysis of Variance (perMANOVA) with 999 permutations was performed on the dissimilarity matrix using *vegan*'s *adonis2* function to determine significant differences in community composition between seasons and locations ([Anderson, 2014](#); [Oksanen et al., 2020](#)), followed by a *post hoc* pairwise analysis (R package *pairwiseAdonis*) ([Arbizu, 2020](#)).

The relationships between fish community data and the measured chemical and physico-chemical variables were examined using a redundancy analysis (RDA) ([Legendre & Legendre, 2012](#)). Prior to this, the combined toxicity of the measured pesticides was estimated using a method developed by [Warne et al. \(2023\)](#) to calculate a multi-substance potentially affected fraction (msPAF) or percentage of species affected. These msPAF values for combined pesticide toxicity were used to represent pesticides in the subsequent analyses (see column "msPAF - Total" in [Supplementary Workbooks 1 and 2](#)). The RDA was performed

using *vegan*'s *rda* function, followed by a stepwise model selection with forward and backward selection based on  $R^2$  and  $P$ -values using *vegan*'s *ordistep* function with 1000 permutations (Legendre & Legendre, 2012; Oksanen et al., 2020). Additionally, a variance partitioning analysis was also performed using *vegan*'s *varpart* function, on variables selected for by the model selection step.

### 3. Results

#### 3.1. Water quality across sampled locations and seasons

The physico-chemical properties of the water column in the 2018 dry and 2020 wet season samples strongly reflected seasonal rainfall patterns. In the three estuaries, salinity shifted from marine/hypersaline (37.5–39 PSU) during the dry season towards being more freshwater influenced (3.4–8.5 PSU) during the wet season, while the Harbour remained marine (shifting from 37.1 to 32.6 PSU) (Supplementary Material, Tables S3 and S4). Turbidity at all locations greatly increased during the wet season (mean turbidity ranging from 0.8 to 13.4 FNU during the dry and 35.5–79.3 during the wet season), as did the concentration of organic matter (DOC and chlorophyll *a*) (Supplementary Material Table S4). Agricultural contaminants mostly increased during the wet season, with higher concentrations of nutrients (N and P) observed in some of the estuaries (Supplementary Material Table S4), and a far greater number of pesticides detected during this season, many at concentrations that exceeded Ecotoxicity Threshold Values (ETVs) for pesticide toxicity (Warne et al., 2023). ETVs utilise the same methods as those used to calculate the Australian and New Zealand Guidelines for Fresh and Marine Water Quality, except that toxicity data for both fresh and marine species is combined (Warne et al., 2018). ETVs that should protect 99% of aquatic species (i.e., PC99, the required level for the mouth of waterways draining into the GBR lagoon) and ETVs that should protect 95% of aquatic species (i.e., PC95, the required level for upstream sites) were both considered (Table 1) (Warne et al., 2023). For non-pesticide variables, national water quality guideline values (WQGVs) were used (ANZG, 2018).

During the dry season (2018), salinity (PSU) in all the sampled locations was indicative of marine waters (>35 PSU, Supplementary Material Table S3). Concentrations of organic matter (chlorophyll *a* and DOC) were markedly higher in the estuaries compared to the Harbour (Supplementary Material Table S3), and in all three estuaries, mean chlorophyll *a* concentrations exceeded the WQGV of 2  $\mu\text{g/L}$  (ANZG, 2018). Concentrations of total Kjeldahl nitrogen (henceforth nitrogen) and total Kjeldahl phosphorus (henceforth phosphorus) were also elevated, exceeding the WQGVs (0.25 mg/L for nitrogen, and 0.02 mg/L for phosphorus) at all four locations (ANZG, 2018). Finally, pesticide

concentrations were consistently below the detection limits, except for trace levels of atrazine, diuron and hexazinone in Sandy Creek and diuron in the Harbour (Supplementary Workbook 1).

During the wet season (2020), there were sharp contrasts in salinity between the Harbour (which remained marine) and the estuaries (which became fresher) (ANOVA:  $F = 12.89$ ,  $P < 0.001$ ; Supplementary Material Table S4). Organic matter (chlorophyll *a* and DOC) was again elevated in the estuaries compared to the Harbour, with DOC concentrations being greatly increased in the estuaries compared to the 2018 dry season (Supplementary Material Table S4). Predictably, Sandy Creek, the most contaminated location historically, stood out from the other locations in terms of its water quality. Overall contaminant concentrations (nutrients and pesticides) were markedly elevated in Sandy Creek compared to the other sampled locations (Table 1, Supplementary Material Table S4). Phosphorus at Sandy Creek exceeded the WQGV >8 times, and nitrogen at both Sandy Creek and Gregory River exceeded the WQGV >3 times (Supplementary Material Table S4). Pesticide concentrations of atrazine, diuron, imidacloprid and metolachlor all greatly exceeded the PC95 level ETVs at Sandy Creek and were extremely high in comparison to the other three locations (ANOVA  $P < 0.01$  in all cases, Table 1). Following Sandy Creek, Gregory River and then the Harbour had the next highest concentrations of pesticides (Table 1). St. Helen's was the only location where none of the detected pesticides exceeded the PC95 ETVs.

#### 3.2. Fish communities

##### 3.2.1. Taxonomic resolution of 12S Tele01 and Elas02 primers

The raw datasets after bioinformatics processing contained 8,746,817 reads for Tele01 and 697,594 for Elas02. After cleaning (before conversion to presence-absence), 4,287,632 reads capturing 180 zOTUs were retained for the Tele01 dataset, and 522,384 reads capturing 15 zOTUs for the Elas02 dataset. Of the 180 zOTUs for Tele01, 81 could be taxonomically resolved to species level, 133 were assigned to the genus level, 157 to family level, and 161 to order. Of the 15 Elas02 zOTUs, 11 were resolved to species level, 13 to the genus, 14 to family level, and all to order.

The Tele01 primer detected 56 families belonging to the classes Actinopterygii and Chondrichthyes across the four locations during both seasons. Of these, 13 families were only detected during the 2018 dry season and six families only during the 2020 wet season (Fig. 2). A total of six families were detected with the Elas02 marker across the four locations and two seasons (Fig. 2). All six families were detected in the dry season, while during the wet season, only three families were observed. Notably, during the wet season, the Tele01 primer set outperformed the Elas02 primers in terms of detecting elasmobranchs. For

**Table 1**

Summary of selected pesticide concentrations sampled in the water column during the 2020 wet season, averaged ( $\pm$ SE) per estuary (2,4-D: 2,4-dichlorophenoxyacetic acid; MCPA: 2-methyl-4-chlorophenoxyacetic acid). A complete listing of all measured pesticides is available in Supplementary Workbooks 1 and 2. Values exceeding default trigger values from water quality guidelines for pesticides at 99% species protection level are in bold text, and those exceeding pesticide Ecotoxicity Threshold Values (ETVs) at 95% species protection are highlighted in grey (Warne et al., 2023).

Pesticide	Gregory (ug/L)	St. Helen's (ug/L)	Sandy Ck (ug/L)	Harbour (ug/L)	ETV - 99% species protection (ug/L)	ETV - 95% species protection (ug/L)	ANOVA	
							F value	P value
2,4-D	0.26 ( $\pm$ 0.02)	0.01 ( $\pm$ 0)	0.2 ( $\pm$ 0.02)	0.06 ( $\pm$ 0.01)	7.3	17	29.75	<0.001
Ametryn	0.01 ( $\pm$ 0)	0.01 ( $\pm$ 0)	0.05 ( $\pm$ 0.01)	0.01 ( $\pm$ 0)	0.079	0.36	35.29	<0.001
Atrazine	<b>0.43</b> ( $\pm$ 0.05)	0.16 ( $\pm$ 0)	<b>2.32</b> ( $\pm$ 0.24)	<b>0.62</b> ( $\pm$ 0.09)	0.27	1.2	39.98	<0.001
Diuron	<b>0.64</b> ( $\pm$ 0.07)	<b>0.13</b> ( $\pm$ 0.02)	<b>2.84</b> ( $\pm$ 0.35)	<b>0.45</b> ( $\pm$ 0.07)	0.075	0.22	34.82	<0.001
Hexazinone	0.56 ( $\pm$ 0.06)	0.04 ( $\pm$ 0)	0.96 ( $\pm$ 0.1)	0.31 ( $\pm$ 0.05)	1.8	2.5	23.3	<0.001
Imazethapyr	0.01 ( $\pm$ 0)	0.01 ( $\pm$ 0)	<b>0.07</b> ( $\pm$ 0.01)	0.01 ( $\pm$ 0)	0.0021 <sup>a</sup>	0.31	50.82	<0.001
Imidacloprid	<b>0.41</b> ( $\pm$ 0.04)	0.01 ( $\pm$ 0)	<b>0.38</b> ( $\pm$ 0.06)	<b>0.16</b> ( $\pm$ 0.03)	0.057	0.13	11.63	<0.001
MCPA	<b>0.07</b> ( $\pm$ 0.01)	0.01 ( $\pm$ 0)	<b>0.12</b> ( $\pm$ 0.02)	<b>0.05</b> ( $\pm$ 0.01)	0.0075	1.5	10.43	<0.001
Metolachlor	<b>0.13</b> ( $\pm$ 0.01)	0.01 ( $\pm$ 0)	<b>0.61</b> ( $\pm$ 0.1)	<b>0.03</b> ( $\pm$ 0)	0.0079	0.4	24.37	<0.001
Metribuzin	0.01 ( $\pm$ 0)	0.01 ( $\pm$ 0)	0.03 ( $\pm$ 0)	0.01 ( $\pm$ 0)	2	2.6	14.66	<0.001

<sup>a</sup> Unpublished ETVs for imazethapyr obtained from M. Warne (personal communication February 26, 2023).

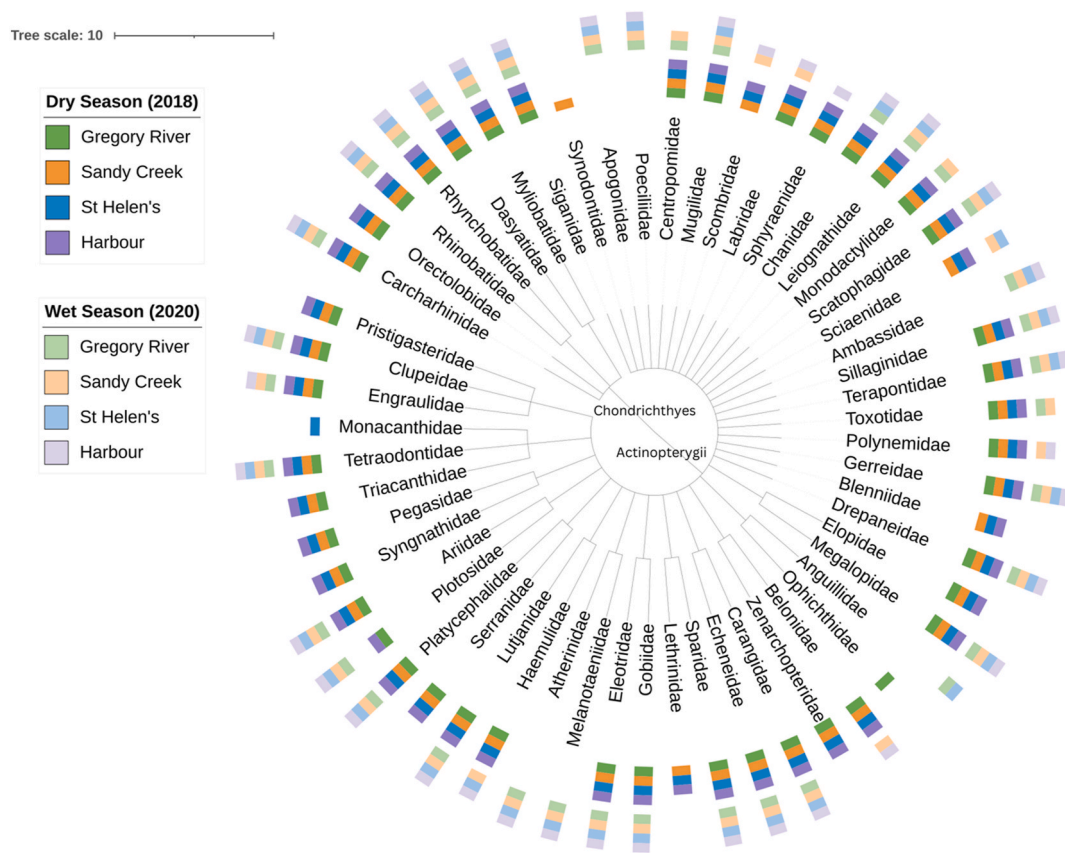


Fig. 2. Family tree depicting teleost (Class Actinopterygii) and elasmobranch (Class Chondrichthyes) families detected at each location during the sampled dry and wet season.

the wet season, we retrieved 23 elasmobranch zOTUs using the Tele01 marker, with a total of 116 occurrences, compared to 15 zOTUs and 17 occurrences with the Elas02 marker (Supplementary Workbook 3). Seventeen species detected in this study were on the IUCN Red List of threatened species, including four critically endangered elasmobranchs (Supplementary Material Table S6) (IUCN, 2021).

### 3.2.2. Taxonomic richness of fishes

The richness of teleost and elasmobranch fishes on a combined

dataset for Tele01 and Elas02 results was significantly lower during the 2020 wet season (115 zOTUs) compared to the 2018 dry (164 zOTUs) (Fig. 3, Table 3). Among the four locations, there were no significant differences in taxonomic richness during the dry season. During the wet season, however, richness was much greater in the Harbour (mean =  $63.5 \pm 6.61$ ) compared to Sandy Creek (mean =  $40.7 \pm 5.85$ ) and St Helens (mean =  $30.7 \pm 3$ ), as well as in Gregory River (mean =  $46.8 \pm 2.4$ ) compared to St Helens (Fig. 3, Table 3; see Supplementary Material Fig. S2 for species richness rarefaction curve).

### 3.2.3. Fish community composition between locations and seasons

The composition of fish communities were markedly different between the two sampled seasons as well as among locations, with a significant interaction observed between season and location (Table 3, Supplementary Material Fig. S3). Within the dry season, fish communities differed among the four locations, with a post-hoc pairwise analysis revealing that this difference was largely between Gregory River and the other 3 locations (Table 3). During the wet season, similarly, fish communities exhibited different compositions among locations, with the addition that all three estuaries also possessed significantly different fish communities to the Harbour (Table 3).

### 3.2.4. Influence of environmental variables, nutrients and pesticides on fish communities

The model selection analysis for a combined dataset with chemical/physico-chemical measurements and fish community data from both sampled seasons (2018 dry and 2020 wet) returned five explanatory variables – salinity, turbidity, nitrogen, pesticides (i.e., msPAF values for pesticide mixtures) and DOC – which collectively explained 40.51% of the variation in the sampled fish communities (Fig. 4a, Table 2). Salinity was the most significant correlate by a large margin, followed by

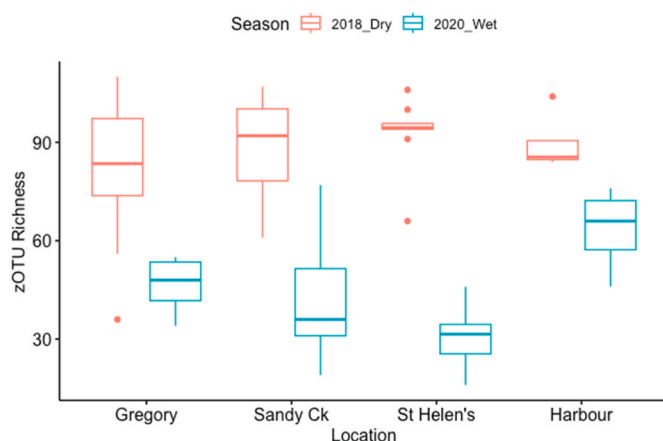
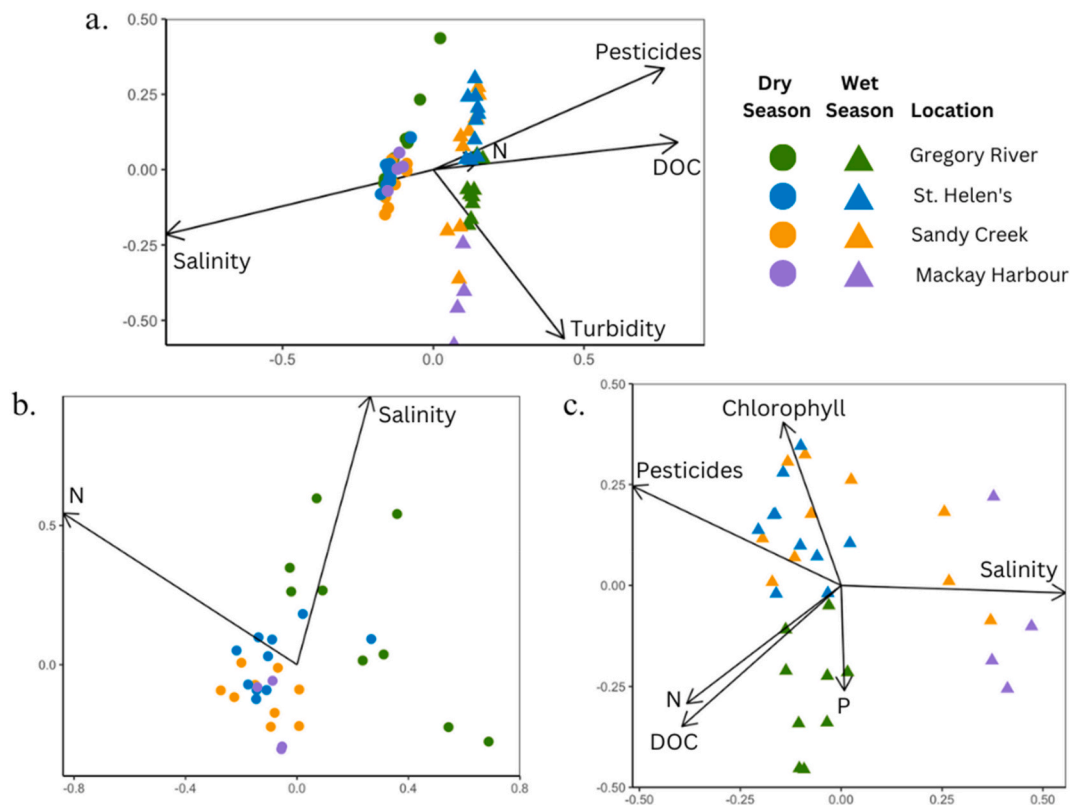


Fig. 3. Comparison of the taxonomic richness of fish communities sampled at the four locations for each season (dry: 2018, wet: 2020) detected using the Tele01 and Elas02 primers. The top and bottom lines of the box represent the first and third interquartile markers (25th and 75th percentiles respectively), and the middle line represents the median richness.



**Fig. 4.** RDA ordination plots examining the relationships between Tele01 fish communities and significantly co-related chemical and physico-chemical variables during (a) both seasons together (2018: dry season, 2020: wet season), (b) dry (2018) and (c) wet (2020) season.

**Table 2**

Summary of chemical and physico-chemical variables contributing to the explained variation in fish community data based on a redundancy analysis performed on the Tele01 dataset during both seasons (dry: 2018; and wet: 2020), dry season (2018) and the wet season (2020). DOC: Dissolved Organic Carbon; N: Total Kjeldahl Nitrogen; P: Total Kjeldahl Phosphorus.

Variable	Both seasons			Dry season 2018			Wet season 2020		
	F value	P value	% contribution to total variance	F value	P value	% contribution to total variance	F value	P value	% contribution to total variance
Chlorophyll <i>a</i>	–	–	–	–	–	–	1.39	0.122	3.71
DOC	1.26	0.209	1.27	–	–	–	2.09	0.006	5.58
N	2.16	0.038	2.18	2.68	0.001	8.04	1.54	0.066	4.12
P	–	–	–	–	–	–	2.34	0.008	6.25
Pesticides <sup>a</sup>	1.50	0.14	1.51	–	–	–	1.95	0.011	5.22
Salinity	27.26	0.001	27.48	1.65	0.021	4.94	2.10	0.008	5.61
Turbidity	8.00	0.001	8.07	–	–	–	–	–	–

<sup>a</sup> Pesticides here refer to the msPAF values calculated for pesticide mixtures.

turbidity and nitrogen (Table 2). The corresponding ordination plot showed that salinity was strongly associated with the dry season communities, while the remaining variables were associated with the wet season communities (Fig. 4a). The same analysis performed for each season separately showed that the variables selected for by our model explained 12.98% and 30.48% of the total variation in the fish community data during the 2018 dry and 2020 wet seasons, respectively (Fig. 4b and c). The variables significantly correlated with the fish community data in the 2018 dry season were salinity and nitrogen (Table 2). From Fig. 4b, salinity was most closely associated with the fish communities at Gregory River and nitrogen with St. Helen's. During the 2020 wet season, phosphorus, salinity, DOC and pesticides (msPAF values) were significantly correlated with fish community structure, with nitrogen and chlorophyll *a* also selected for by the model (Table 2). As indicated by the ordination plot (Fig. 4c), pesticide and chlorophyll *a* concentrations appeared to correlate with fish communities in Sandy Creek and St Helens, while nutrients (N and P) and DOC were associated

with Gregory River, and salinity aided in discriminating fish communities in the marine-dominated harbour sites. The variance partitioning analysis for both years collectively showed that 19.6% of the seasonal variation in the fish community data could be explained by pesticides, and 34.9% of the variation was explained by the remaining variables selected for by our model. Pesticides were not selected for during the dry season, but within the wet season, 2.3% of the inter-location variation in fish communities was attributed to pesticides and 11.4 % to the remaining variables.

#### 4. Discussion

We examined tropical estuarine fish communities exposed to different levels of agricultural run-off during a wet and a dry season. As predicted, overall, fish richness reduced significantly from the 2018 dry to the 2020 wet season, and community composition was markedly different. Salinity stood out as the most significant co-relate for this

**Table 3**  
ANOVA results table for species richness and community composition analyses.

	Dataset	Terms for comparison	F value	P value		
<b>Taxa Richness</b>	Both seasons	Locations	1.97	ns		
		Seasons	164.73	2.00E-16		
	Dry season 2018	Location:Season	4.24	0.0087		
		Locations	0.84	ns		
	Wet season 2020	Locations	6.83	0.001		
	Wet season 2020	Gregory - Sandy Ck	Gregory - St Helens	0.08	ns	
			Gregory - Harbour	0.26	0.04	
		Sandy Ck - St Helens	Gregory - Harbour	18	ns	
			St Helens - Harbour	0.15	ns	
		Sandy Ck - Harbour	Sandy Ck - St Helens	0.27	0.026	
			St Helens - Harbour	0.37	0.0008	
		<b>Community Composition</b>	Both seasons	Locations	2.58	0.008
				Seasons	46.68	0.001
			Dry season 2018	Location:Season	2.65	0.002
Locations				2.84	0.001	
Wet season 2020	Locations		2.5	0.001		
	Dry season 2018		Gregory - Sandy Ck	4.35	0.001	
Gregory - St Helens			3.69	0.001		
Gregory - Harbour			1.53	0.003		
Sandy Ck - St Helens		1.27	ns			
Sandy Ck - Harbour		1.53	ns			
St Helens - Harbour		2.06	0.024			
Wet season 2020	Gregory - Sandy Ck	Gregory - St Helens	2.33	0.014		
		Gregory - Harbour	2.59	0.001		
	Sandy Ck - St Helens	Gregory - Harbour	5.01	0.001		
		St Helens - Harbour	1.2	ns		
	Sandy Ck - Harbour	Sandy Ck - St Helens	2.32	0.031		
		St Helens - Harbour	3.58	0.001		

inter-seasonal change in fish community composition, with turbidity and nitrogen also contributing to the observed variation. Variance partitioning showed that pesticides were responsible for a large proportion of the variation as well. Within the sampled dry season, differences in fish communities among locations were not very pronounced, with a relatively low proportion of variation being explained by the selected variables (salinity and nitrogen) and species richness being similar across locations. Within the wet season, however, inter-location differences in fish communities were significantly associated with pesticide and phosphorus loadings, along with environmental variables (salinity and DOC). There was also a sharp contrast in species richness between the estuaries and the Harbour, where seasonal changes were less marked, reflecting its stable marine conditions.

#### 4.1. Effect of environmental variables on the detected fish communities

We observed pronounced differences in fish community richness and composition between the 2018 dry and 2020 wet seasons, with 156 out

of the 180 observed taxa being detected during the dry season, versus 108 taxa during the wet season. Estuarine fish assemblages are known to undergo seasonal shifts in composition and have more variable species richness compared to stable freshwater or marine environments (de Morais & de Morais, 1994; de Moura et al., 2012), with salinity in estuarine systems being correlated with diversity (Barletta et al., 2005).

In total, 73 taxa were detected exclusively during the 2018 dry season – with these being dominated by marine elasmobranchs and teleosts from the families Mugilidae, Dasyatidae, Gobiidae, Carcharhinidae and Clupeidae; while 24 taxa were observed exclusively during the 2020 wet season, with families Eleotridae, Terapontidae and Poeciliidae dominating. Unsurprisingly, salinity was one of the strongest correlates to these changes. Salinity is a key driver in shaping coastal fish communities, with marine and freshwater fishes each possessing different mechanisms of osmoregulation (McKinley et al., 2011; Dolbeth et al., 2016; Araújo et al., 2017). All four sampled systems were characterized by marine to hypersaline conditions during the dry season, followed by a drop to more freshwater-influenced conditions in the estuaries after heavy rainfalls. In contrast to the estuaries, taxonomic richness was relatively stable in Mackay Harbour, where there was little seasonal fluctuation in salinity. Consequently, a range of pelagic marine taxa were sampled in the Harbour, e.g. the eagle ray (*Aetobatus* sp.), barracuda (*Sphyraena* sp.) and Australian spotted mackerel (*Scomberomorus munroi*).

Other non-contaminant stressors (turbidity and DOC) also contributed to the observed differentiation in fish assemblages. Several studies have shown that turbidity can alter fish community composition (Khan & Ali, 2003; Candolin et al., 2008; Shetty et al., 2015). For example, high turbidity in tropical rivers in southern India has been linked to reduced species richness, as compared to more clear waters which favour algal growth, providing an increase in food availability for benthic feeders (Shetty et al., 2015). In the present study, turbidity exceeded WQGVs at all locations during the 2020 wet season (Supplementary Workbook 2), with this being most pronounced at St. Helen's (~20-times > WQGV) (ANZG, 2018). Dissolved organic carbon (DOC) (also elevated during the wet season) arises from decomposed organic matter, i.e. dead plant and animal material, may be negatively correlated to fish productivity and is also associated with high turbidity (Craig et al., 2017).

A potential bias in this study was that the volume of water filtered per sample was reduced during the wet season due to clogging of filters from high turbidity. However, turbid water contains more sedimentary particles with the capacity to adsorb eDNA, which can potentially increase eDNA detection (Díaz et al., 2020). Thus, despite a lower volume of water being filtered, one may expect a high eDNA detection rate in turbid samples. For example, Kumar et al. (2021) found ~3.2-times higher eDNA yield in the same volume of turbid water when compared to clear waters due to the higher concentrations of suspended organic and inorganic material. In the present study, we filtered the full volume of water on a subset of wet season samples and compared these to samples from the same sites during the dry season. We found that detections still remained higher during the less turbid dry season (see Supplementary Material Fig. S1), supporting the finding that richness is greater in these systems during the marine-dominated dry season and is not driven by turbidity or the volume of filtered water.

#### 4.2. Effect of agricultural contaminants on detected fish communities

Our study emphasises how agricultural run-off during the wet season markedly increases nutrient (nitrogen and phosphorus) and pesticide loadings within estuaries. Consequently, the observed variation in fish community structure was also associated with these. Inter-seasonal changes in fish communities were significantly correlated with nitrogen, and notably, within the wet season, pesticides (msPAF values for pesticide mixtures) and phosphorus were significant contributors to the variation in fish communities among locations. Atrazine was detected at



concentrations up to 3 µg/L at Sandy Creek (Supplementary Workbook 2), which is > 2-times over the PC95 ETV and >8-times over the PC99 ETV (Warne et al., 2023). At present, knowledge of the ecotoxicological effects of pesticides is mostly derived from single-species laboratory experiments. For example, exposure to 2 µg/L of atrazine for 24 and 48 h has been shown to inhibit the activity of biotransformation and antioxidant enzymes in the liver of the tropical fish *Prochilodus lineatus*, as well as cause DNA damage (Santos & Martinez, 2012). In the context of this study, the detection of 3 µg/L atrazine in Sandy Creek suggests the potential for greater health consequences in the fish of this region.

A range of pesticides were shown to co-occur during the wet season, while most pesticides were below detection or at very low concentrations during the dry season. Mean concentrations of diuron at all four locations during the wet season were >0.1 mg/L, with a maximum concentration of 3.9 mg/L detected at Sandy Creek (Table 2). These concentrations pose a potential risk to fish, with similar concentrations being found to cause estrogenic effects in sexually mature female Nile tilapias (*Oreochromis niloticus*) (Pereira et al., 2016). Atrazine and diuron, along with several other pesticides detected in this study, are photosystem II inhibiting herbicides, which function by disrupting photosynthesis (Murata et al., 2007; Huggins et al., 2017). This may cause indirect effects to fish communities by impacting aquatic plants and phytoplankton which serve as their food sources. High concentrations of atrazine can cause significant decreases in phytoplankton biomass, leading to cascading detrimental effects on zooplankton and other higher trophic levels (DeNoyelles et al., 1982; Starr et al., 2017). In addition to herbicides, the present study also detected the insecticide imidacloprid, which, similarly, has been experimentally shown to decrease zooplankton abundance and the survival of zooplanktivorous fish populations (Yamamoto et al., 2019).

During the wet season, nutrient concentrations were particularly elevated in Sandy Creek, exceeding the WQGVs (PC99) by up to 3-times and 72-times for nitrogen and phosphorus respectively (ANZG, 2018). In other coastal regions, nutrient concentrations similar to those observed here (particularly at Sandy Creek) have been known to greatly increase turbidity, cause seagrass die-offs, and alter the community structure of pelagic and demersal fish species (Caddy, 2000; Richardson & Zimba, 2002). Elevated nutrient concentrations have been correlated to increased biomass and trophic diversity of fishes due to the associated increase in productivity and prey abundance (Nixon & Buckley, 2002; Breitburg et al., 2009; Warry et al., 2016). Frey et al. (2011) found that indicators of highly nutrient-enriched waters commonly included fishes that were capable of withstanding low levels of dissolved oxygen. However, beyond a certain breakpoint concentration, the number of fish decreased as nutrient concentrations increased (Frey et al., 2011).

Historically, Sandy Creek is known to have high loads of agricultural run-off at concentrations which can impair the ecological health of estuarine systems (Wallace et al., 2016a; Huggins et al., 2017). Despite having the smallest catchment area, Sandy Creek is one the largest sources of photosystem II inhibiting pesticide yield to the GBR catchment (Turner et al., 2012; Wallace et al., 2015; Wallace et al., 2016a; Huggins et al., 2017). We also found relatively high concentrations of pesticides in the Gregory River. This is despite the system's loadings steadily declining over the last decade (Newham et al., 2017; Healthy Rivers to Reef Partnership, 2021). Interestingly, Gregory River had the most distinct fish assemblages during the dry season and included all the detected critically endangered species, e.g., the scalloped hammerhead (*Sphyrna lewini*) (Supplementary Material Table S6).

The changes observed in this study emphasise the complexity of estuarine systems, where biota are exposed to highly variable mixtures of natural and anthropogenic stressors that change over space and time. We detected a mixture of contaminants which typify agricultural runoff and are indicative of the anthropogenic pressures typical of urbanized coastlines, yet, the interacting effects between multiple stressors on coastal communities are still rarely investigated (Johnston et al., 2015; Mayer-Pinto et al., 2015; O'Brien et al., 2019). Evidence for the chronic

effects of individual pesticides do not account for interactive effects which may occur from mixtures of multiple pesticides, inert compounds in pesticides, physico-chemical variables present in natural environments, and even pathogens (Clifford et al., 2005; Smith et al., 2012). Such mixtures may produce unpredictable and highly variable effects on different species, being potentially lethal for some while having no effect on others (Relyea, 2009). Given that real-life contaminated environments unequivocally host mixtures of pesticides and other compounds in a variety of environmental conditions (Warne et al., 2020), there is a strong need to investigate the interacting effects of contaminants on biodiversity.

## 5. Conclusion

The impacts of agricultural run-off on the Great Barrier Reef's coastal fish communities have been overlooked to date, despite close proximity to sources. As highlighted in this study, coastal fish communities within the region are being shaped by complex interactions between natural (e.g., salinity) and anthropogenically derived variables (e.g., nutrients and pesticides), with this being most evident during the wet season when loadings from agricultural run-off are at their peak. Given the influence of agricultural run-off, and additional pressures such as fishing, climate change, tourism, development and habitat modification, there is a pivotal need for baseline data to monitor the trajectories of fish communities. As indicated here, eDNA metabarcoding has the capacity to efficiently and effectively provide occurrence data on a wide breadth of fish taxa, and importantly, produce ecological data which is responsive to both natural and anthropogenic stressors.

## CRediT authorship contribution statement

**Aashi Parikh:** Formal analysis, Writing – original draft, Writing – review & editing. **Johan Pansu:** Conceptualization, Methodology, Writing – review & editing. **Adam Stow:** Supervision, Writing – review & editing. **Michael St J. Warne:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Writing – original draft, Writing – review & editing. **Christine Chivas:** Data curation, Resources. **Paul Greenfield:** Data curation, Formal analysis, Software, Writing – original draft, Writing – review & editing. **Frédéric Boyer:** Conceptualization, Formal analysis, Methodology, Software. **Stuart Simpson:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Rachael Smith:** Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – review & editing. **Jacob Gruythuysen:** Data curation, Project administration, Writing – review & editing. **Geoffrey Carlin:** Formal analysis, Investigation, Project administration, Writing – review & editing. **Natalie Caulfield:** Investigation, Methodology. **Frédérique Viard:** Conceptualization, Writing – review & editing. **Anthony A. Chariton:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Supplementary data is publicly available on Dryad: <https://doi.org/10.5061/dryad.ttdz08m16> and other supplementary information on Zenodo: <https://doi.org/10.5281/zenodo.8368904>

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2024.123954>.

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