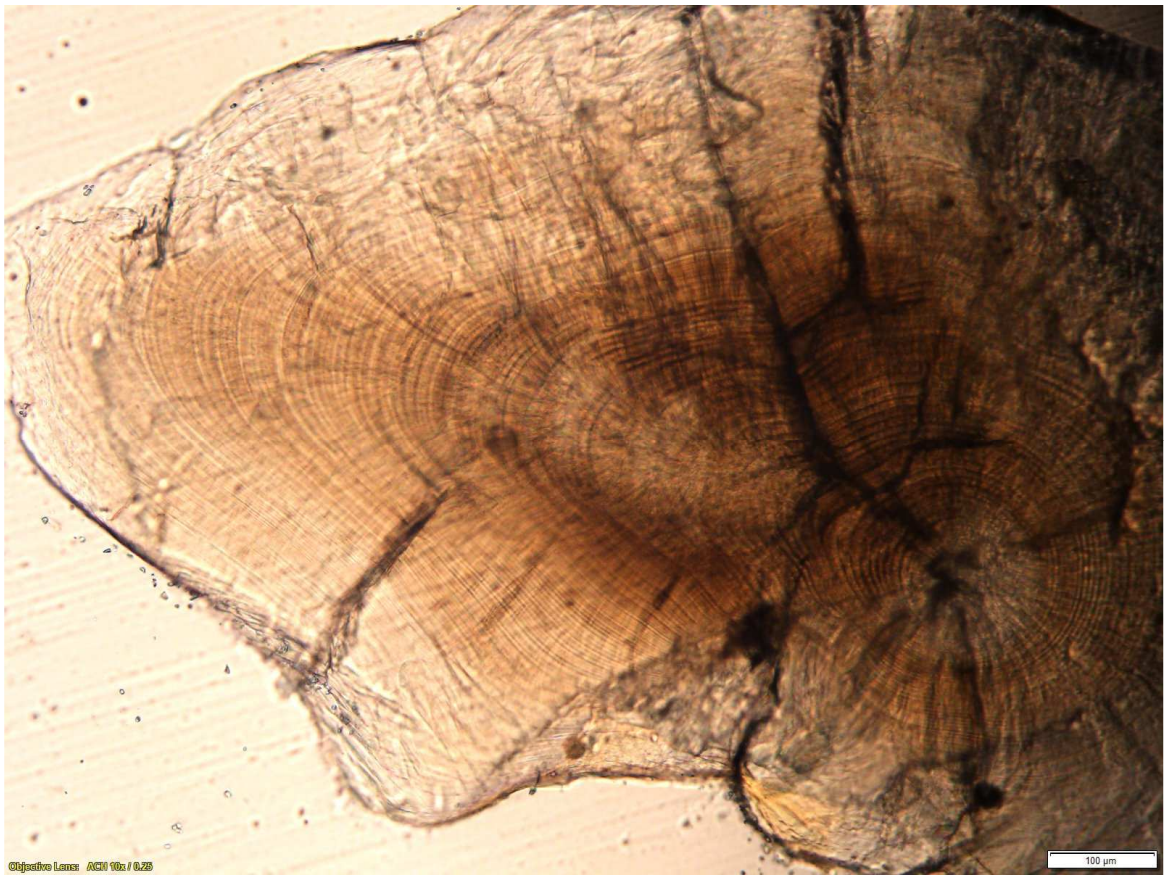
Department of Primary Industries

Monitoring fish in the Koondrook-Perricoota Forest Watering Event 2014: Otolith ageing and microchemistry component

Meaghan Duncan, David Hohnberg, Kate Martin and Peter Graham



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Non-technical summary

Monitoring fish in the Koondrook-Perricoota Forest Watering Event 2014: Otolith ageing and microchemistry component

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Objectives

1. Identify whether common carp spawn and recruit in Koondrook-Perricoota Forest in response to environmental flows and whether they subsequently colonise the Murray River.
2. Identify if native species (carp gudgeon) spawn and recruit within Koondrook-Perricoota Forest in response to environmental flows.

Key words

Otolith, microchemistry, daily ageing, Koondrook-Perricoota Forest, common carp, carp gudgeon.

Summary

Koondrook-Perricoota Forest (KPF) is located on the Murray River floodplain upstream of Barham in NSW. It spans approximately 33,000 hectares and is one of six The Living Murray icon sites due to its significant wetland and creek ecosystems. These ecosystems are highly reliant on floodwaters given flooding was historically more common than under current regulated conditions. Recently, a range of environmental works and measures have been completed that enable the KPF to be flooded without the need for an overbank flow. This can potentially have a range of positive and negative outcomes for fish populations (and for other environmental values such as the River Red Gum community in the forest). The creation of shallow wetland habitat may provide ideal spawning conditions for native small-bodied fish. Similar conditions are also favoured by the introduced common carp and there is a risk that inundating a large area of wetland and creek habitat that this species will spawn and recruit in large numbers. Consequently, KPF could potentially act as major a source population of common carp that could colonise the Murray River following environmental watering.

The water management structures were first operated in August 2014 and the spawning and recruitment response of native carp gudgeon and common carp within KPF was evaluated. Given that water from the Murray River was used to flood KPF, it is important to be able to distinguish between fish that were spawned within KPF and those that were spawned outside KPF and washed in during inflows (either managed flows or natural overbank flows). To distinguish between common carp spawned in KPF and common carp spawned elsewhere, it is possible to examine the chemical structure of the otoliths – bony structures inside the inner ear – given these are known to retain the chemical signature of water at the fish's birth place. This requires sampling larval fish both within KPF and at comparison sites outside of the forest to

characterise the chemical signature of different spawning sites. Unfortunately, very few larval fish were captured during October and November 2014. However, as there were no natural inflows into KPF following completion of the event in September 2014, it was possible to calculate the date of birth of young-of-year fish sampled in March 2015 by counting the number of rings on their otoliths. If the date of hatch was after the completion of the inflows, that fish was considered to have originated in the KPF and to have successfully recruited to YOY age. Results indicated that both species successfully spawned and recruited with common carp ranging in age from 26-116 days and carp gudgeon ranged from 65-85 days old.

The chemical composition of the otoliths from common carp from KPF was analysed in order to characterise the chemical signature of KPF at different time periods. This was achieved by analysing the core of the otolith to identify the chemical composition during the fish's larval stage, and at the edge of the otolith to identify the chemical composition when the fish was several months old. Results indicated that the core and edge otolith chemical composition was different, suggesting that the KPF water chemistry is not temporally stable and changes within a matter of months. This will have implications for future work where the aim is to assign older fish collected from the Murray River back to their birth place.

Introduction

The inundation of a river's floodplain is important for the conservation of biotic diversity and the production of plant and animal biomass, including fish (Junk *et al.* 1989). Riverine fish in the Murray-Darling Basin (MDB) are susceptible to the effects of river regulation as many species have been shown to utilise off-channel habitat (Conallin *et al.* 2011a; Lyon *et al.* 2010). For some species, floodplain inundation provides an opportunity to spawn and then connections to the river allow the juvenile fish to disperse back into the river. Developing technology in the otolith microchemistry field is enabling researchers to gain a detailed understanding of the migration history of a fish, including its place of hatch, by analysing the chemical composition of its otolith (Elsdon *et al.* 2008). Otoliths (fish earstones) are found in all teleost fishes and are important for balance and/or hearing (Campana 1999). Otoliths are formed by the daily accumulation of layers of calcium carbonate from before hatch to death and are therefore commonly utilised for ageing fish. Another important characteristic of otoliths is that they are metabolically inert, thus they retain the chemical composition of the environment they were in at the time each layer was deposited (Campana 1999). Therefore, the chemical composition of the otolith reflects the water chemistry at the time that the layer was deposited. It is possible to analyse different layers within the otolith so that a fish's migration history can potentially be determined – if there are differences in water chemistry among sites. For example, otolith microchemistry analysis was able to demonstrate that Barmah-Millewa Forest is a key spawning ground for common carp (C*yprinus carpio*), and, when conditions are suitable, it is the source of most young-of-year common carp captured downstream at Torrumbarry Weir (Crook & Gillanders 2006; Macdonald & Crook 2014).

The Koondrook-Perricoota Forest (KPF) is a large floodplain forest located adjacent to the Murray River in southern New South Wales and is one of The Living Murray (TLM) icon sites, containing significant wetland and creek ecosystems. A range of environmental works and measures have been implemented in order to enable watering of the forest without relying on an overbank flow. There are many fish objectives relating to the flooding and these are described in detail in Duncan et al. (2015). Two objectives relate specifically to spawning and recruitment of common carp and native fish; Objective 1: the operation of the scheme will not result in dispersal of common carp spawned in KPF to the Murray River and, Objective 8: the operation of the scheme will result in a beneficial impact on the fish community in KPF.

Objective 1: Common carp

Common carp are an important invasive species in the Murray-Darling Basin that could potentially benefit from environmental watering that inundates their preferred spawning and recruitment grounds. This species is known to invade floodplain environments where spawning can take place (Conallin *et al.* 2012; Gilligan in prep; Jones & Stuart 2009; Macdonald & Crook 2014; Stuart & Jones 2006). They have a well-documented invasion history, are capable of exploiting a wide range of environments and can quickly respond to suitable conditions through rapid spawning and recruitment in large numbers (Koehn 2004). Given that common carp are known to spawn in shallow floodplain habitats along the Murray River, it is likely that inundating the KPF floodplain will provide conditions suitable for common carp spawning, provided watering occurs during their peak spawning period of approximately August to November. Juvenile common carp may then seek to leave KPF via outflows from the return channel (RC) or Barber Creek regulators (BCR) where they may ultimately contribute to the biomass of common carp in the Murray River (Gilligan in prep; Macdonald & Crook 2014). It is therefore important to collect data on common carp spawning and recruitment in KPF and the nearby rivers to provide data to managers on how best to operate the structures to avoid this risk, such as avoiding watering in peak spawning months.

Objective 8: Native fish

One of the main objectives of flooding KPF for fish is to improve recruitment of large and small–bodied native species (Hohnberg *et al.* 2015). The inundation of water into KPF will potentially encourage the recruitment of some native fish and would allow native fish the opportunity to move naturally among wetland pools and to the Murray River via the return channel, the fishways on the inlet channel or Swan Lagoon. To address this objective, carp gudgeon *(Hypseleotris spp.)* were used as a model species given they are a common, widespread, easily sampled and known to spawn and recruit in isolated waterbodies (Gilligan *et al.* 2009). Environmental watering of KPF will greatly increase the habitat available to this species and spawning and recruitment is likely to occur, provided water quality remains within appropriate thresholds. It is possible that these fish may seek to move back to the main river channels when connection reoccurs (Conallin *et al.* 2011b; Lyon *et al.* 2010) and thus the KPF may be an important nursery ground for this species.

Capturing larval common carp and carp gudgeon in KPF will not provide unequivocal evidence these species spawned within KPF given larval fish could have washed into the forest during environmental watering. And similarly, young-of-year (YOY) common carp captured in the Murray River adjacent to KPF may not necessarily originate from the forest. Consequently, the approach taken here is to first calculate the fish's age using it's otolith to determine if spawning occurred either before (in remnant water holes), during inflows or after the 2014 event ceased (Elsdon *et al.* 2008). Those individuals confirmed to have hatched within the KPF will then be subjected to otolith microchemistry analysis to determine if there is a unique chemical signature of the forest. Young-of-year fish can then be collected from the Murray River and the chemical signature of the core of the otolith compared to that of the KPF fish to determine if there is a match and subsequently whether the fish originated from spawning in the forest (Crook *et al.* 2013; Macdonald & Crook 2014). This method relies upon larval fish also being collected from as many comparison locations as possible in order to increase the confidence that YOY fish can be assigned to their natal site.

Methods

Larval sampling

Sampling for larval fish commenced after the environmental watering had ceased in October 2014 in order to increase the likelihood that larval fish that were sampled were the result of spawning within the forest and not the Murray River or other sites upstream. Three one week sampling trips were conducted between the 20/10/14 to the 28/11/14. We attempted to collect larval and juvenile common carp (<4 week old) from nine sites within KPF as well as seven comparison sites outside of KPF (Appendix 1). Sampling was also conducted in the Goulburn River, Broken River and Ovens River by other researchers. Some otoliths from larval common carp were collected from the Broken and Ovens Rivers, but these were not identified in time to be included in the current analysis. Larval fish were sampled using a combination of drift nets, quatrefoil light traps and boat trawls. No set sampling methodology was used as the goal was to sample a minimum of 15 larval fish of each species at each site, but preferably 30 larval common carp. Typically, five light traps were randomly set at dusk along the littoral edge at each site and retrieved early the following morning. The entire sample from the light trap was preserved in 100% ethanol in the field and brought back to the laboratory for processing. Fish were identified to species level and classified by developmental stage as either larvae (yolk-sac larva, protolarvae, flexion, post flexion, metalarvae) or juvenile/adults (Serafini & Humphries 2004).

Ageing carp gudgeon

Unfortunately, very few common carp or carp gudgeon larvae were collected either within KPF or at comparison sites in October-November 2014. Thus the original planned otolith microchemistry analysis to determine differences across nursery sites could not be completed. However, there was no connectivity between KPF and the Murray River and downstream sites following completion of inflows. Therefore, ageing YOY fish caught in early 2015 could confirm if common carp and carp gudgeon spawned and recruited in KPF following completion of the event. Consequently, follow-up sampling of YOY common carp and carp gudgeon was conducted in conjunction with the 2015 condition monitoring. Daily ageing was then used to determine the fish's date of hatch and therefore whether it was the result of a spawning event pre– or post–environmental watering. If the fish was confirmed to have been the result of a spawning after the completion of inflows, then it was considered to have successfully spawned and recruited (to YOY age) in KPF. These fish were sampled using standard SRA techniques as detailed in the report (Duncan & Graham 2016).

YOY carp gudgeon specimens were sent to the South Australian Research and Development Institute (SARDI) for daily increment counts in otolith microstructure. Carp gudgeon were measured to the nearest millimetre and sagittal otoliths were removed. Otoliths were mounted individually in Crystalbond™, proximal surface downwards, and polished down to the primordium using a graded series of wetted lapping films (9, 5, and 3 µm). Sections were examined using a compound microscope (x 400) fitted with a digital camera and cellSens image analysis software. Increments were counted blind with respect to fish length and capture date. The numbers of rings (usually an estimate of age in validated species) were determined by counting the number of increments from the primordium to the otolith edge. Three successive counts were made by two readers for one otolith from each fish. If these differed by more than 10% the otolith was rejected, but if not, the mean was used as an estimate of the number of increments. The daily formation of increments has not been validated for this species so data has been presented as an average number of rings. If increments are formed daily, spawn dates would be determined by subtracting the estimated age from the capture date. We propose to validate the daily formation of increments in carp gudgeon in the near future.

Ageing common carp

Daily increment counts in otolith microstructure were examined in YOY common carp. Common carp YOY were measured to the nearest millimetre and lapilli otoliths were removed. The same

procedure to count the otolith increments was then followed as described for carp gudgeon. Increment counts were considered to represent true age of juvenile common carp (Vilizzi 1998) and spawn dates were determined by subtracting the estimated age from the capture date.

Otolith microchemistry analysis

Despite there being no connectivity to the Murray River following the 2014 event, there was still the possibility that common carp that originated in KPF following the event could colonise the Murray River in subsequent floods. Thus it was considered important to investigate the applicability of the microchemistry technique to characterise the signature of KPF in 2016, and to also determine if the signature was temporally stable from October 2014 to March 2015. These data will then be used to guide future otolith microchemistry work.

Ageing analysis was used to verify that the common carp were spawned after inflows into the KPF ceased (or within a few days). The otoliths from these fish were sent for microchemistry analysis at the University of Adelaide. We did not include common carp from comparison sites given that these fish may have dispersed from their natal location and thus the chemical signature from the core of their otolith may not be representative of their capture location.

The concentration of elements in the otolith (^{24}Mg, ^{55}Mn, ^{88}Sr, ^{138}Ba) was determined using a New Wave UP213 nm UV laser operated in Q-switch mode connected to an Agilent 7500cs inductively coupled plasma-mass spectrometer (ICP-MS). ^{43}Ca was also analysed as an internal standard, and enabled the element:Ca ratio to be calculated, and ^{115}In (Indium) was analysed so that the otolith material could be distinguished from epoxy resin. Ablation of the otolith material occurred in a sealed chamber, with the ablated sample gas being extracted from the chamber and transported to the ICP-MS through a smoothing manifold in an argon and helium gas stream. Prior to data collection, background concentrations of all elements in the sample chamber were measured for 30s and subtracted from the sample signal.

A 250 µm element profile, following the curvature of the daily growth increments, was analysed on the outside edge of the otoliths (referred to hereafter as the edge). Element concentrations were averaged across the profile, and represents the most recent otolith growth and corresponds to the location in which the fish were collected. For the young (30-day old) fish, these element signals were used to define a natal signature (referred to hereafter as the core). In the larger, older fish (approx. 3 months old: collected in March 2015) a second 250 µm element profile was analysed in the region that corresponded to the natal signatures of the 30-day old fish.

A reference standard (National Institute of Standards and Technology, NIST 612) was measured after every 10 ablations to correct for instrument drift throughout each session. A calcium carbonate standard (MACS-3, United States Geological Society) was also measured at the beginning and conclusion of each session. Precision estimates for individual elements, measured as the mean relative standard deviation (RSD) were less than 5% for all elements. The concentration of each element was standardised to ^{43}Ca. The element:Ca ratio (expressed as mmol·mol^{-1}) was calculated by converting the element counts to mmols, and then dividing the element in mmols by Ca (mols).

Statistical analysis microchemistry.

As microchemistry analysis was only performed on common carp from KPF, the only statistical analysis that could be done was to determine if there were any temporal differences in the microchemical signatures from late 2014 when most common carp were spawned to March 2015 when they were collected as young-of-year.

Data were analysed in the software package PERMANOVA+ (Anderson *et al.* 2008). This package allows the analysis of multivariate data using permutation to test for significant differences between groups. Data were imported into the software package PRIMER-E (Clarke & Warwick 2001) and log(x+1) transformed. Euclidian distances were used to generate the dissimilarity matrix, which formed the basis of subsequent analyses. Patterns in the data were visualised using Principal Coordinates Ordination (PCO). A one factor PERMANOVA was

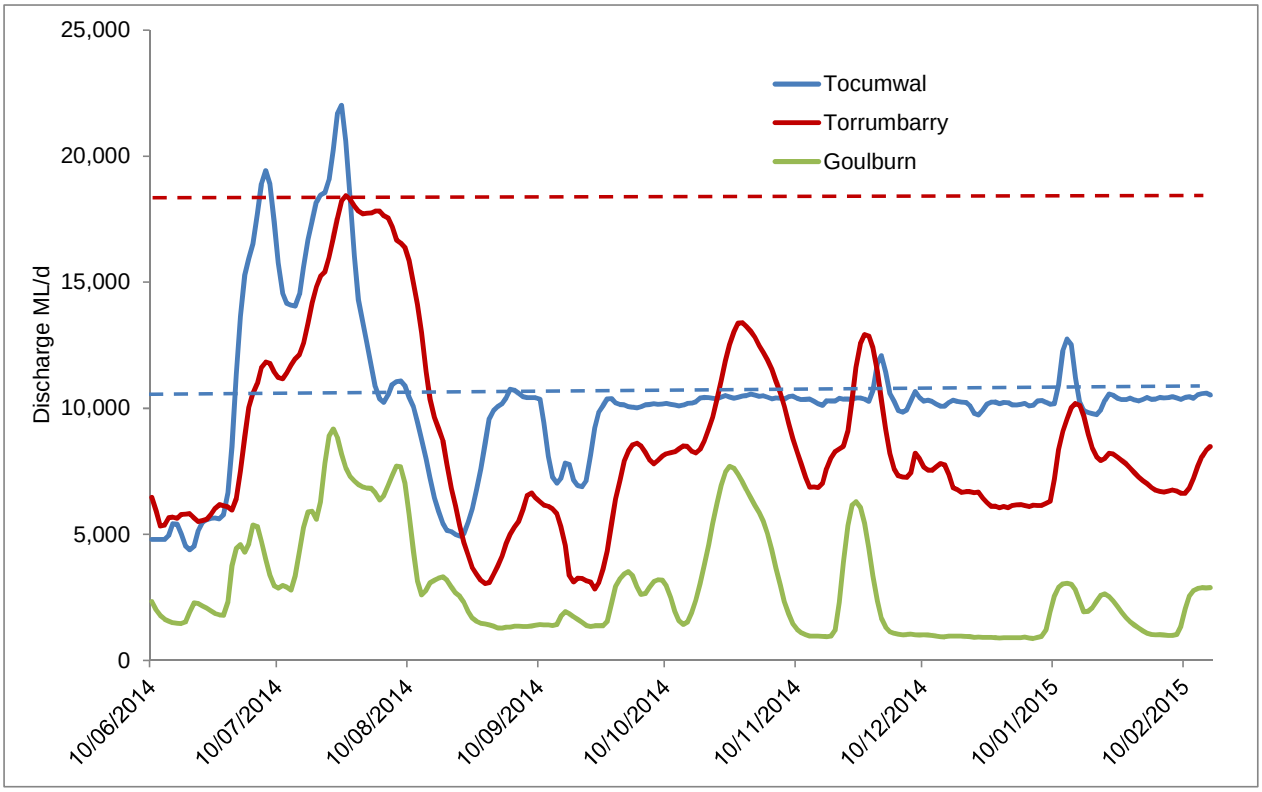
conducted to determine if there were differences between the otolith microchemistry between the core (i.e. soon after hatching) to the edge of the otolith (i.e. the date of collection). All fish were included with the exception of cypcar 32, 34 and 35 given these were larval fish and only had an edge sample (the edge sample of these fish is actually considered a core sample given it is so close to the primordium of the otolith). A second one factor PERMANOVA was then conducted to determine if there were statistically significant differences in the otolith microchemistry of core samples based on month. Edge samples were not included as they were all collected from March and thus any significant differences that may have occurred could be due to either temporal changes, or due to sampling the edge of the otolith only. A follow-up pair-wise PERMANOVA was conducted to see which months were significantly different. Comparisons involving fish that did not have a known month corresponding to their core microchemistry data (due to missing ageing data) were removed from the analysis. For core samples, the month was assigned based on the month of hatch as determined by ageing. For the edge samples, it was simply the month they were collected. Significance of results was calculated using permutation and Monte Carlo *P*-values were used when the number of available permutations was <100 (Anderson *et al.* 2008). A *P*-value <0.05 was considered significant.

Results

Hydrology

The 2014 environmental watering event began on the 14th of August and ended on the 29th of September. The volume of water released was not enough to enable the return channel to operate, and flows into Barber Creek were so low that there was no connectivity to the Wakool River. The vertical-slot fishway remained open until 8th October. Theoretically, there was an opportunity any larval common carp that may have been present in late September to have made their way out of the forest into the Murray River via the vertical slot fishway. However, this is highly unlikely given common carp larvae and juveniles <1 month post-hatch have a poor swimming ability and tend to disperse via downstream drift (Gilligan & Schiller 2003; Mills 1991). Following the completion of the event, there was no further connection to the Murray or surrounding rivers at the time of YOY sampling in early 2015 (Figure 1). Upstream at Tocumwal, there was flooding at Barmah-Millewa Forest from early July to mid August 2014 (Figure 1).

Figure 1 Mean daily discharge at Tocumwal and Torrumbarry. Dashed blue line represents approximate onset of floodplain inundation at Tocumwal and dashed red line indicates approximate onset of inundation at Swan Lagoon.



Sampling results

There were only six larval common carp caught in KPF while more 2660 larval and juvenile carp gudgeon were collected in KPF and comparison sites following the 2014 watering. A total of 104 YOY common carp and 322 carp gudgeon were collected in March 2015.

Ageing

Otoliths were successfully removed from five larval common carp, 30 YOY common carp and 34 YOY carp gudgeon. Common carp ranged in size from 16–87 mm. Age for common carp could be determined from the number of increments for 32 individuals (Figure 2) (Vilizzi 1998). The otoliths were lost for three individuals and thus their age was not determined. The common carp ranged in age from 26–116 days, placing the date of hatch from the 26th of September 2014 until the 15th of January 2015 (Table 1). Carp gudgeon ranged in size from 20-27 mm and had 65-85

increments on their otoliths placing their date of hatch 16th of December 2014 and the 4th of January 2015 (

Table 2). However, given that the ageing method has not been validated for this species, the date of hatch should be interpreted with a degree of caution. Nevertheless, the estimated date of hatch of these fish was greater than two months after the completion of inflows, thus it is reasonable to assume that these fish were spawned within KPF.

Figure 2 Images of lapilli otoliths from juvenile common carp showing the daily increments.

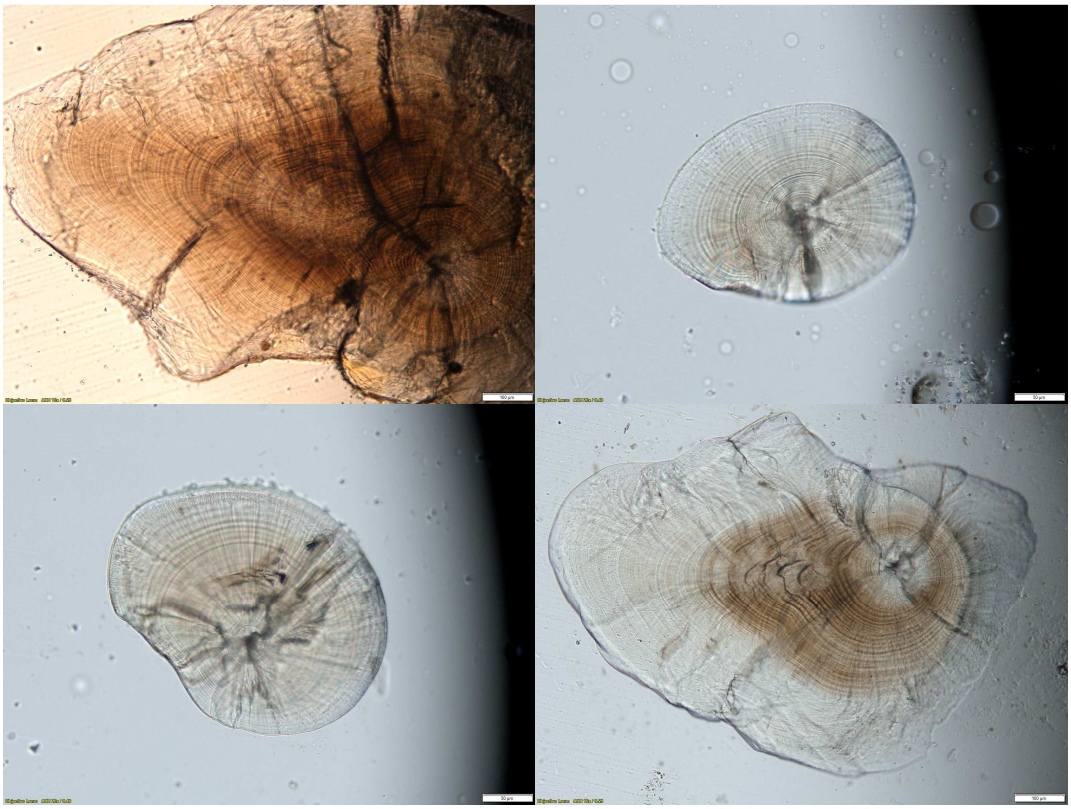


Table 1 Common carp ageing results.

Fish ID	Sample ID	Date of collection	Fork length (mm)	Age (days)	Date of hatch
cypcar 01	235	25/03/2015	68	119	26/11/2014
cypcar 02	233	12/03/2015	55	107	24/11/2014
cypcar 03	233	12/03/2015	56	104	28/11/2014
cypcar 04	228	21/10/2014	20	27	24/09/2014
cypcar 05	234	4/03/2015	87	116	8/11/2014

cypcar 06	234	4/03/2015	80	108	16/11/2014
cypcar 07	232	11/03/2015	40	75	26/12/2014
cypcar 08	232	11/03/2015	41	74	27/12/2014
cypcar 09	232	11/03/2015	37	72	29/12/2014
cypcar 10	232	11/03/2015	35	60	9/01/2015
cypcar 11	232	11/03/2015	39		
cypcar 12	232	11/03/2015	36	66	4/01/2015
cypcar 13	232	11/03/2015	38	70	30/12/2014
cypcar 14	232	11/03/2015	34	63	7/01/2015
cypcar 15	232	11/03/2015	32	54	15/01/2015
cypcar 16	232	11/03/2015	35		
cypcar 17	232	11/03/2015	42	70	30/12/2014
cypcar 18	232	11/03/2015	40		
cypcar 19	232	11/03/2015	37	73	27/12/2014
cypcar 20	232	11/03/2015	34	65	5/01/2015
cypcar 21	232	11/03/2015	48	81	19/12/2014
cypcar 22	232	11/03/2015	37	60	10/01/2015
cypcar 23	232	11/03/2015	38	61	9/01/2015
cypcar 24	232	11/03/2015	36	63	7/01/2015
cypcar 25	232	11/03/2015	48	77	24/12/2014
cypcar 26	232	11/03/2015	37	61	9/01/2015
cypcar 27	232	11/03/2015	36	63	6/01/2015
cypcar 28	232	11/03/2015	38	65	5/01/2015
cypcar 29	232	11/03/2015	42	67	3/01/2015
cypcar 30	232	11/03/2015	35	66	4/01/2015
cypcar 31	232	11/03/2015	39	63	7/01/2015
cypcar 32	227	22/10/2014	16	29	23/09/2014
cypcar 33	227	22/10/2014	16	26	26/09/2014*
cypcar 34	227	22/10/2014	18	28	24/09/2014*
cypcar 35	227	22/10/2014	18	26	26/09/2014*

*These fish are referred to as October in subsequent PCO graphs and PERMANOVA analysis.

Table 2 Carp gudgeon ageing results.

Fish ID	Date of collection	Total length (mm)	No. of rings (increments)	Estimated date of hatch based on one ring per day (not validated for this species)
hypsp 01	11/03/2015	23	82	18/12/2014
hypsp 02	11/03/2015	22	83	18/12/2014
hypsp 03	11/03/2015	20	85	16/12/2014
hypsp 04	11/03/2015	23	72	29/12/2014
hypsp 05	11/03/2015	21	72	28/12/2014
hypsp 06	11/03/2015	24	80	21/12/2014
hypsp 07	11/03/2015	20	70	31/12/2014
hypsp 09	11/03/2015	24	83	18/12/2014
hypsp 10	11/03/2015	21	72	29/12/2014
hypsp 11	11/03/2015	25	74	27/12/2014
hypsp 12	11/03/2015	20	71	29/12/2014
hypsp 13	11/03/2015	23	74	27/12/2014
hypsp 14	11/03/2015	23	74	27/12/2014
hypsp 15	11/03/2015	26	69	1/01/2015
hypsp 16	11/03/2015	23	77	24/12/2014
hypsp 17	11/03/2015	25	75	26/12/2014
hypsp 18	11/03/2015	20	74	26/12/2014
hypsp 19	11/03/2015	22	74	26/12/2014
hypsp 20	11/03/2015	23	68	2/01/2015
hypsp 21	11/03/2015	22	65	4/01/2015
hypsp 22	11/03/2015	25	72	29/12/2014
hypsp 23	11/03/2015	25	79	22/12/2014
hypsp 24	11/03/2015	25	73	28/12/2014

hypsp 25	11/03/2015	23	76	24/12/2014
hypsp 26	11/03/2015	23	66	4/01/2015
hypsp 27	11/03/2015	24	80	20/12/2014
hypsp 28	11/03/2015	27	85	15/12/2014
hypsp 29	11/03/2015	24	78	23/12/2014
hypsp 31	11/03/2015	26	66	4/01/2015
hypsp 32	11/03/2015	26	69	1/01/2015
hypsp 33	11/03/2015	24	68	2/01/2015
hypsp 34	11/03/2015	26	84	16/12/2014

Microchemistry results

Microchemistry analysis of the edge of the otolith corresponding to the date and location collected was carried out on 28 common carp. A further 25 common carp otoliths were also analysed at the core of the otolith at a region that corresponded to fish's natal period Table 3. PCO indicated two main groups corresponding to the core and edge samples (Figure 3). The three larval fish that had only their edge of their otolith analysed (corresponding to their natal signature) were most similar to the core samples of the other larger fish. PERMANOVA analysis indicated the differences between the core and edge multi-elemental signatures apparent in the PCO were significantly different (pseudo-F = 29.56, $P < 0.0001$). PCO indicated that there was some differentiation of multi-elemental signatures of the core of the otoliths in different months (Figure 4) and PERMANOVA supported this (pseudo-F = 3.53, $P < 0.005$). Follow-up pairwise analysis indicated that October and December and January and October were significantly different (Table 4).

Table 3 Raw common carp microchemistry data. Data was collected for either the core of the otolith and/or the edge of the otolith depending on the size of the fish. * These were larval fish so while the edge of the otolith was examined, this is comparable to the core region on other fish.

Location on otolith	Fish ID	Mg24 mmol.mol	Mn55 mmol.mol	Sr88 mmol.mol	Ba138 mmol.mol
EDGE	cypcar 01	0.0593	0.0042	2.0019	0.0533
CORE	cypcar 01	0.0718	0.0018	1.7178	0.0235
EDGE	cypcar 05	0.0637	0.0022	1.9090	0.0897
CORE	cypcar 05	0.1058	0.0010	1.4339	0.0230
EDGE	cypcar 06	0.0585	0.0014	1.6809	0.0612
CORE	cypcar 06	0.0718	0.0013	1.6891	0.0253
EDGE	cypcar 08	0.0446	0.0008	1.4797	0.0667
CORE	cypcar 08	0.0687	0.0083	1.6031	0.0270
EDGE	cypcar 09	0.0352	0.0004	1.4344	0.0421

CORE	cypcar 09	0.0720	0.0025	1.5662	0.0172
EDGE	cypcar 10	0.0366	0.0004	1.5235	0.0661
CORE	cypcar 10	0.0868	0.0026	1.7275	0.0272
EDGE	cypcar 11	0.0351	0.0012	1.2128	0.0472
CORE	cypcar 11	0.0768	0.0023	1.5884	0.0182
EDGE	cypcar 12	0.0309	0.0006	1.5064	0.0515
CORE	cypcar 12	0.0887	0.0034	1.6479	0.0264
EDGE	cypcar 13	0.0327	0.0003	1.5328	0.0575
CORE	cypcar 13	0.1122	0.0023	1.5570	0.0246
EDGE	cypcar 15	0.0331	0.0007	1.6905	0.0527
CORE	cypcar 15	0.0826	0.0044	1.5424	0.0186
EDGE	cypcar 16	0.0353	0.0013	1.4304	0.0409
CORE	cypcar 16	0.0713	0.0019	1.5270	0.0167
EDGE	cypcar 17	0.1329	0.0018	1.4555	0.0616
CORE	cypcar 17	0.0694	0.0013	1.5080	0.0178
EDGE	cypcar 18	0.0357	0.0005	1.5895	0.0741
CORE	cypcar 18	0.0556	0.0009	1.6584	0.0337
EDGE	cypcar 19	0.0340	0.0004	1.4061	0.0506
CORE	cypcar 19	0.0835	0.0022	1.6910	0.0256
EDGE	cypcar 20	0.0300	0.0003	1.4994	0.0622
CORE	cypcar 20	0.0775	0.0020	1.7176	0.0205
EDGE	cypcar 21	0.0386	0.0004	1.4504	0.0413
CORE	cypcar 21	0.0787	0.0009	1.5557	0.0169
EDGE	cypcar 22	0.0382	0.0004	1.3792	0.0642
CORE	cypcar 22	0.0822	0.0024	1.5698	0.0216
EDGE	cypcar 23	0.0277	0.0003	1.3937	0.0399
CORE	cypcar 23	0.0918	0.0056	1.5543	0.0214
EDGE	cypcar 24	0.0354	0.0008	1.5616	0.0777
CORE	cypcar 24	0.0685	0.0022	1.6656	0.0217
EDGE	cypcar 25	0.0382	0.0006	1.7637	0.0873
CORE	cypcar 25	0.0836	0.0027	1.6415	0.0232

EDGE	cypcar 27	0.0298	0.0009	1.3173	0.0459
CORE	cypcar 27	0.0716	0.0023	1.5270	0.0207
EDGE	cypcar 28	0.0254	0.0007	1.3460	0.0560
CORE	cypcar 28	0.0894	0.0043	1.4976	0.0174
EDGE	cypcar 29	0.0765	0.0009	1.5777	0.0723
CORE	cypcar 29	0.0799	0.0013	1.6634	0.0244
EDGE	cypcar 30	0.0911	0.0009	1.4688	0.0752
CORE	cypcar 30	0.0817	0.0018	1.4581	0.0197
EDGE	cypcar 31	0.0303	0.0003	1.7236	0.0621
CORE	cypcar 31	0.1048	0.0027	1.5227	0.0236
EDGE*	cypcar 32	0.1027	0.0004	1.4781	0.0134
EDGE*	cypcar 34	0.3242	0.0006	1.4373	0.0099
EDGE*	cypcar 35	0.1920	0.0005	1.6453	0.0149

Figure 3 PCO analysis for the core and edge samples. The three larval fish with edge samples only (equivalent to the core of larger fish) are indicated by the black triangles.

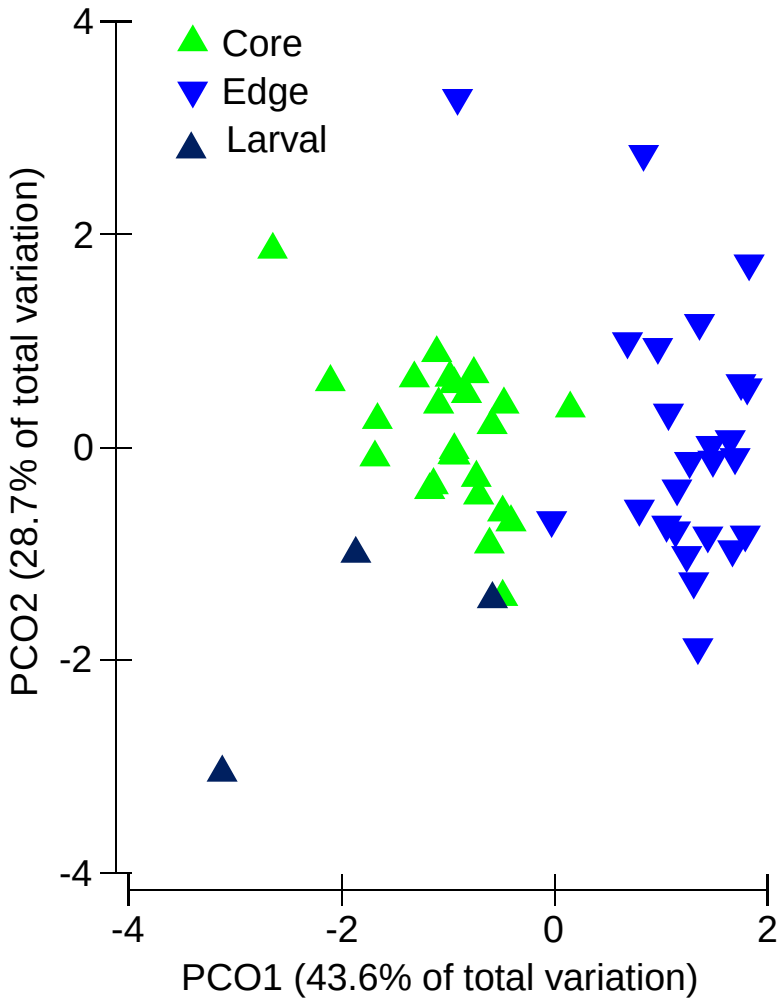


Figure 4 PCO of core samples only by month. The sampling month of some core samples is unknown due to unsuccessful ageing and these have been omitted from the analysis.

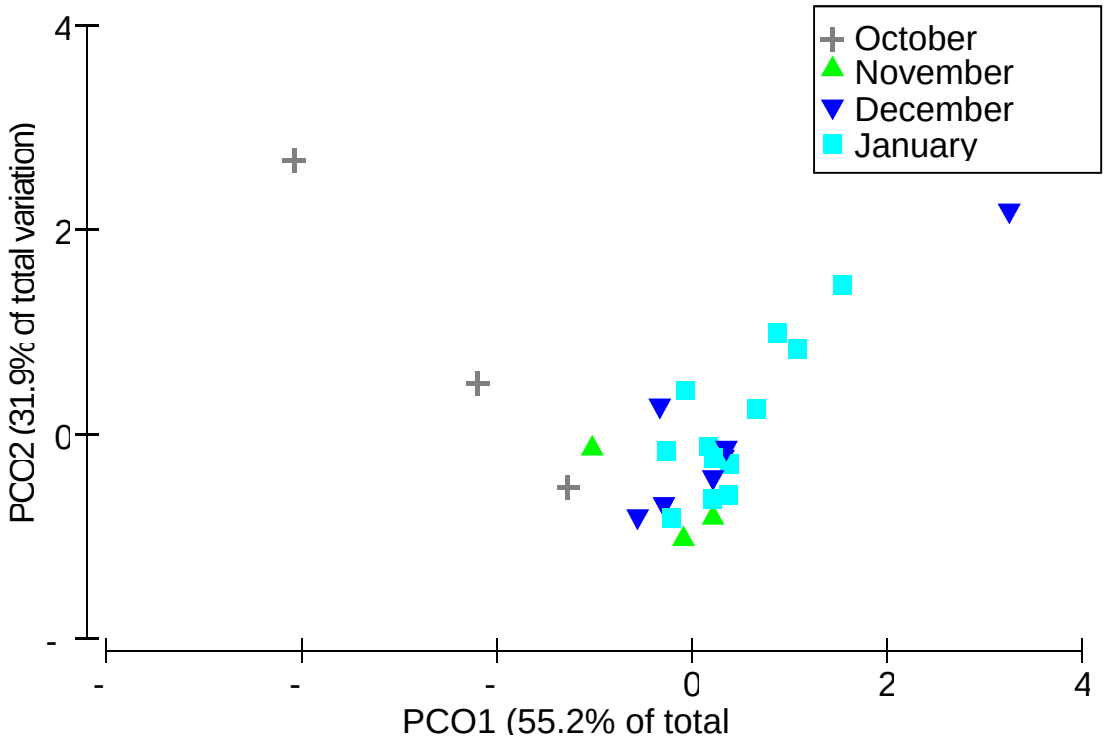


Table 4 Pairwise PERMANOVA comparisons of core microchemical signatures by month.

Groups	t	*P* (perm)	Unique perms	*P* (Monte Carlo)
November, December	0.90703	0.5017	120	0.4329
November, January	1.478	0.1237	455	0.13
November, October	1.7739	0.2032	10	0.1237
December*, January	0.11283	0.9979	9042	0.9972
December*, October	2.3445	**0.0081**	120	**0.0158**
January, October	3.5033	**0.0021**	455	**0.0004**

*December fish were hatched in late December, placing them closer to the January fish in age than the November fish, see Table 1.

Discussion

Common carp

Otolith microchemistry can be a powerful tool for identifying natal sites for common carp. However, the technique is dependent on there being distinct chemical signatures at different natal sites. This has previously been demonstrated for common carp in the vicinity of KPF including Barmah-Millewa and several nearby Victorian tributaries (Crook & Gillanders 2006; Macdonald & Crook 2014). While the current study was limited by a lack of samples at comparison sites, the data generated provides valuable information that will be used to inform future sampling. We have demonstrated that the otolith chemical signature of common carp between the core and edge of common carp that had spent their entire lives within KPF were not consistent. The variation in otolith chemical signatures is typically due to differences in water chemistry, though water temperature and salinity may also impact on otolith chemical composition (Elsdon & Gillanders 2003). It is not unusual for otolith chemical signatures to change over time. For example, elemental concentrations in common carp otoliths across four years were not stable at all sites examined in a study surrounding the Barmah-Millewa region (Macdonald & Crook 2014) and the Lachlan River region (Crook *et al.* 2013).

Despite the differences between core and edge samples, closer inspection of the core data only revealed no significant differences in core microchemistry for most of the spawning period. There was a significant difference between core signatures for fish analysed from December and October, however, the December fish were all hatched in late December and so in effect much closer to the early January cohort. The only other significant difference was between January and October. Thus it is clear that the chemical signature that was deposited at the core of the otoliths began to change around late December, coinciding with the beginning of summer and therefore greater evaporation from waterbodies. A much greater change in microchemistry was apparent in the edge sample taken in March, providing further evidence that changes in water chemistry brought about by evaporation may have had an effect on otolith microchemistry.

The implications of the change in elemental signatures over time in KPF are that it will be necessary to collect larval common carp annually to characterise the chemical signature of the forest. In addition, larval common carp would ideally be collected from throughout the spawning season given the temporal variation observed in this study. It will only be possible to match young-of-year fish caught in the Murray River to their natal site if the postlarval common carp were collected from the same cohort.

This study was limited by the lack of common carp larvae caught at comparison sites. Therefore, the low numbers of larvae meant that it could not be determined if KPF had a distinct chemical signature in 2014. The lack of common carp larvae upstream of KPF is interesting given that Barmah-Millewa Forest experienced a flood from early July to mid-August and the lower Goulburn River also experienced a minor flow peak in late July (Figure 1). It is possible that the common carp upstream of KPF responded quickly to the increased flows and had already spawned and possibly dispersed prior to sampling from late October. However, the Barmah-Millewa Fish condition monitoring project samples larval fish annually during October and November in the Murray River around Barmah-Millewa Forest and common carp are consistently caught at one site (Murray River at Morning Glory) while they are less frequently sampled at the Barmah Choke and Ladgroves Beach (Raymond *et al.* 2014). The current project also sampled at Morning Glory and used drift nets of the same design as those used for the Barmah-Millewa project. It is recommended that future sampling commences in late August, particularly following winter watering, to maximise the chance that larval common carp are captured.

Carp gudgeon

Following the 2014 event there was no subsequent connection to the Murray River. Given that ageing analysis put the date of hatch of this species as December and January, we can be confident that carp gudgeon successfully spawned and recruited within KPF following the environmental watering event. Daily ageing alone is sufficient to answer the spawning and

recruitment questions for this species – provided there is no subsequent connection to the Murray River. If there was connectivity, otolith microchemistry analysis could be conducted in the same way as for common carp to confirm recruitment in KPF. However this will require sufficient sampling effort to provide a clear understanding of the level of variability of the otolith and water isotope signatures.

Carp gudgeon are a generalist species – they are tolerant of a broad range of conditions and are capable of spawning and recruiting in isolated waterbodies (Gilligan *et al.* 2009). It would be useful to extend the ageing work to other species found in KPF that are not quite as abundant including Australian smelt (*Retropinna semoni*), Murray-Darling rainbowfish (*Melanotaenia fluviatlis*) and flathead gudgeon (*Philypnodon grandiceps*). This would be relatively straightforward if there was no connectivity to the Murray River following the event as daily ageing is all that would be required. This would then provide additional evidence of the value of flooding KPF to small-bodied native species.

Conclusions and recommendations

Daily ageing of carp and carp gudgeon demonstrated that the inaugural environmental watering event using the new water management infrastructure provided suitable conditions for common carp to spawn and recruit within KPF. Consequently, there is a real risk that KPF could act as a major source population for common carp to disperse into the Murray or Wakool Rivers if there is connectivity following a flood. Fortunately, following the 2014 event there was no risk of common carp YOY colonising surrounding rivers given there was no subsequent connection between the forest and the Wakool or Murray Rivers. The forest was almost totally dry by March 2016 and this resulted in almost all common carp perishing. Conversely, most native fish also perished and any initial positive recruitment response of carp gudgeon was subsequently lost given there was no subsequent lateral connectivity that allowed movement between the KPF and the Murray or Wakool Rivers.

To maximise the benefits to native fish, annual watering is desirable in KPF to ensure habitats do not totally dry out and to allow fish to move out of the forest to colonise the Murray and Wakool Rivers. This needs to be balanced with the risks of triggering a mass common carp spawning event.

Otolith microchemistry results for common carp indicated that the chemical signature of the KPF changes over a period of months, most likely due to evaporation altering the water chemistry. This means that in future sampling events larval samples will need to be obtained across the entire spawning season to ensure YOY can be successfully matched to their source population. When data is available from right across the spawning season, it will provide a definite understanding of natal origin of any larvae sampled. Such information on the original of larvae, and related information from length-frequency data from condition monitoring, will support the adaptive management of the forest and planning for future environmental watering events to optimise the achievement of the objectives for native fish in KPF.

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Appendices

Appendix 1

Appendix1 Light trap, drift net and boat trawl sampling sites.

Stream	Site	KPF or comparison site	Latitude	Longitude	Larval carp (number sent for microchemistry analysis)	Larval/juvenile carp gudgeon collected	YOY carp (number aged)	YOY carp gudgeon (number aged)
Penny Royal Creek	Penny Royal Lagoon	KPF	-35.75462	144.36325	0	76	16	31 (28)
Barbers Creek	Barbers Creek No.1	KPF	-35.64878	144.20480	0	0	0	1
Barbers Creek	Barbers Creek No.2	KPF	-35.60275	144.20692	0	1	13	22
Murray River	Barmah Lake	Comparison	-35.94952	144.95776	0	96	0	0
Boundary Lagoon	Boundary Lagoon	KPF	-35.73917	144.33542	0	743	2 (2)	27
Myloc Creek	Boysons	KPF	-35.71662	144.33331	4 (4)	0	0	0
Clarkes Creek	Clarkes Lagoon	KPF	-35.79087	144.42444	0	439	0	40
Murray River	Moira Lake	Comparison	-35.94559	144.93167	0	19	0	0
Murray River	Morning Glory	Comparison	-35.08046	144.94534	0	0	0	2
Myloc Creek	Myloc No.2	KPF	-35.69850	144.27869	0	34	2 (2)	23
Pothole Creek	Pothole Creek	KPF	-35.75425	144.40591	2 (2)	587	0	2
Murray River	Return channel	Comparison	-35.69693	144.21718	0	7	0	1

Barbers Creek	Sandy Bridge	Comparison	-35.50822	144.07719	0	1	0	23
Thule Creek	Thule Creek (downstream of regulator)	Comparison	-35.60856	144.30733	0	399	0	5
Thule Creek	Thule Creek Regulator	Comparison	-35.69629	144.33862	0	32	0	0
Myloc Creek	Myloc 4	KPF	-35.71490	144.30400	0	0	25 (25)	4 (4)
Burrumbury Creek	BC 2	KPF	-35.74965	144.35709	0	0	1 (1)	11
Belbins Creek	Belbins 1	KPF	-35.7447	144.3570	0	0	0	20
Barbers Creek	Nelsons	KPF	-35.6795	144.2457	0	0	20	17
Horseshoe Lagoon	Horseshoe Lagoon	KPF	-35.8510	144.4013	0	0	0	11
Long Lagoon	Long Lagoon	KPF	-35.6115	144.2292	0	0	0	20
Smokehouse Lagoon 1	Smokehouse 1	KPF	-35.6273	144.2490	0	0	0	20
Smokehouse Lagoon 2	Smokehouse 2	KPF	-35.6316	144.2471	0	0	0	20
McMahons Creek	McMahons	KPF	-35.6654	144.2698	0	0	25	16
Myloc Creek	Sandys Crossing	KPF	-35.6932	144.2658	0	226	0	0
Murray River	Tocumwal	Comparison	-35.8171	145.56146	0	0	0	6