Stable Isotopes Approach to Infer the Feeding Habit and Trophic Position of Freshwater Fishes in Tropical Lakes

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A B S T R A C T

A study was conducted on the stable isotope variation of muscle tissues from selected freshwater fish species from Temenggor Lake and Chenderoh Lake, Perak, Malaysia from December 2014 to March 2015. The objective is to assess the stable isotopes of $\delta^{13}C$ to identify the carbon sources and $\delta^{15}N$ of fish species from Temenggor and Chenderoh Lake, Perak, Malaysia to infer the trophic position of these fishes. Four types of fish species were analyzed by using stable isotope approach which were Hampala barb (*Hampala macrolepidota*), Oxygaster cyprinus (Oxygaster anomalura), Peacock Bass (*Cichla ocellaris*) and Nile Tilapia (*Oreochromis niloticus*). Stable isotopes of $\delta^{13}C$ and $\delta15N$ were analysed using an elemental analyser Thermo Finnigan Flash EA 2000 connected to Finningan delta v avantage plus isotope ratio mass spectrometry by a ConFlo II interface. The $\delta^{13}C$ values from both lakes implies a C3 phytoplankton as reported from the literature. Based on $\delta^{15}N$ values of fish species, *O. anomalura* occupies the highest trophic level in Temenggor Lake, reflecting its carnivorous feeding habit despite its small size while *C. ocellaris* was the highest in Chenderoh Lake, implying its predatory behaviour. Further analysis should be carried out to incorporate primary producers and consumers to elucidate the food web in the tropical lake ecosystem. This study provides a reference record for conducting stable isotopes in the food web of tropical lake ecosystem for better management and deeper understanding of the ecosystem functioning.

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INTRODUCTION

Gut content analysis, fecal analysis, and direct observation are common methods use for assessing food webs both in the field and in the laboratory [1, 2]. This method has been useful to identify the diet of a species over a short time frame, but is not always a good approach of what it might have been eating on a regular basis [3, 4].

Stable isotope analysis is emerging as an important tool for identifying animal diet, trophic position, and movement including highly migratory and marine species [5, 6]. This is vital to ensure the conservation of natural resources in a sustainable way. The application of stable isotopes analysis in environmental science provides a standard tool to trace pollution sources within an environment, to understand the underlying processes of the ecosystem nutrient cycling in both terrestrial and marine systems and provides further understanding of the ecosystem functioning. In the ecology of freshwater tropical lakes, stable isotopes provide further insight to trace food web by providing a longer term portrayal of the diet of a fish within a food web and can be used to identify the trophic position of the fish [7]. The ability to elucidate the food web is critical to the implementation of appropriate management, conservation and restoration efforts.

Isotopes are atoms of a common element that share the same number of protons and electrons, but differ in number of neutrons [8]. Differences in the number of neutron create different atomic masses, which can be measured with an isotope ratio mass spectrometer. Stable isotopes, unlike radioactive isotopes, have combinations of protons and neutrons that are resistant to decay over time. Stable isotopes of different elements

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transfer through food webs with fairly consistent trophic level alterations, providing a natural tracer of food web linkages [5]. This isotopic separation between consumer tissue and diet is known as a discrimination or fractionation factor [8]. Stable isotope values reflect an integrated diet over a time scale determined by the metabolic activity of the tissue and organism analyzed [5].

Carbon isotopic compositions have been used to give information about the carbon sources of production and for determining the energy flow through the food web [9]. ^{13}C is the heavier isotopes and ^{12}C is the lighter isotopes based on the periodic table of the stable isotopes [8]. Heavy stable isotopes are rare and comprise a small percentage of total natural abundance for a given element, but are useful as tracers of ecological processes. There is 98.892 % ^{12}C and only 1.108% of ^{13}C abundance in carbon, which are the stable isotopes commonly used in ecological research. Enrichment in carbon isotopic ratios ($^{13}C/^{12}C$) caused a more positive values while depletion of $^{13}C/^{12}C$ ratios had more negative values. Measurement of carbon stable isotope ratios ($^{13}C/^{12}C$, henceforth referred to as $\delta^{13}C$) is useful to indicate feeding and carbon flow pathways because different energy sources can have distinct $\delta^{13}C$ values [10]. The change in $\delta^{13}C$ values from prey to predator or known as trophic fractionation exhibit little or no trophic level enrichment (<1‰) are useful for identifying the sources of production for consumers in lakes.

Nitrogen isotopic compositions can provide information about the trophic levels in a food web and the processing of nitrogen and sources of nitrogen such as sewage[5,7]. Stable nitrogen isotopes are ^{14}N (lighter elements) and ^{15}N (the heavier nitrogen isotopes) with an abundance of 99.635 % and only 0.365%, respectively [8]. The nitrogen isotope ratios ($^{15}N/^{14}N$; $\delta^{15}N$) of animals become enriched in ^{15}N relative to their food with top predators having the highest concentration of this stable isotopes [11].

Fish are important consumers in auatic food webs and the use of stable isotopes to study the trophic interactions have mostly focused in temperate ecosystems [12, 3,7]. Recently, a study by Zulkifli et al. [9] have used stable isotope markers ($\delta^{13}C$ and $\delta^{15}N$) to determine the food web in a tropical mangrove ecosystem, located at a coastal area of Selangor, Malaysia. To date, most ecological study of fishes in Malaysia focused on the distribution and diversity of the available fishes at the selected sampling sites [13, 14]. To the best of our knowledge, this is the first study on lake food webs using stable isotopes in this region. Therefore, in the present study, we analyzed the carbon and nitrogen isotope ratios in two different fish species from two tropical lakes, Temenggor and Chenderoh

Lake, in Perak, Malaysia. The objective is to identify the potential primary production sources supporting the lake consumers (i.e fish) from the $\delta^{13}C$ values and the trophic position of these fisheries from the $\delta^{15}N$ values. This information is important to further understand the community dynamics of the food web for better management of these natural resources.

MATERIALS AND METHODS

Sampling area
Samplings of fish were carried out in December 2014 to March 2015 at Temenggor Lake and Chenderoh Lake in Perak. The locations of the sampling area are shown in Fig. 1. Temenggor Lake is (15200 hectares) is the second largest lake in Peninsula Malaysia after Kenyir Lake in Terengganu, Malaysia. This man-made lake is located south of 1,533 m high Ulu Titi Basah peak, in Hulu Perak district in the state of Perak. The lake is located about 45 km from the Hulu Perak district capital, Gerik. It was created upon the completion of the Temenggor Hydro Electricity Dam in 1978. Temenggor Lake is categorized at medium state or mesotrophic based on its biological productivity and allowable nutrient loadings, corresponded to Carlson's trophic state index values. However, Chenderoh Lake is categorized as bad or eutrophic [15,16]. Chenderoh Lake is a natural lake, located near the royal town of Kuala Kangsar and archaeological site of Lenggong.
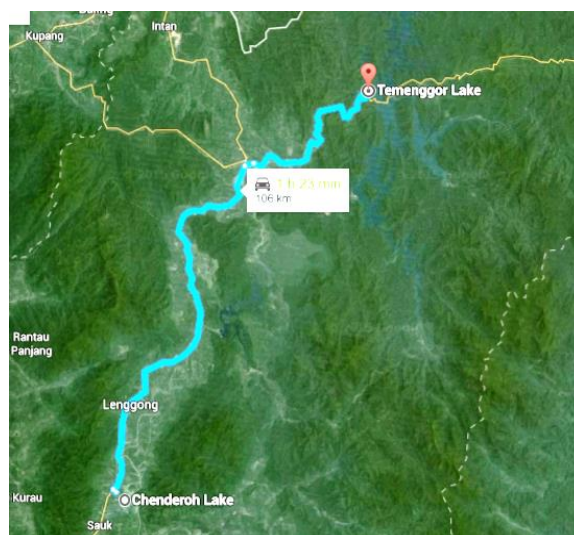
Figure 1. Sampling area of Temenggor Lake and Chenderoh Lake (Google Earth view).

Sample Collection
Conductivity, pH and total dissolved solids (TDS) of the surface water were measured in situ using a field meter,

HACH sension while dissolved oxygen was measured using YSI 5000 dissolved oxygen meter. Nitrate, nitrite and ammoniacal nitrogen were measured by using Hach DR 2010 Spectrophotometer. Fish samples were caught using cast nets with a mesh size of approximately 2.5 cm. The morphology of the fishes were measured; weight, total length and standard length. Three fish species were caught at Temenggor Lake; Hampala barb (*Hampala macrolepidota*), Cyprinus oxygaster (*Oxygaster anomalura*) and Nile tilapia (*Oreochromis niloticus*) while two species were captured in Chenderoh Lake; Peacock Bass (*Cichla ocellaris*) and Hampala barb (*Hampala macrolepidota*).

Stable Isotopes Analysis

Two fish from each species were dissected in the laboratory and only the muscles were used for $\delta^{13}C$ and $\delta^{15}N$ analyses. Swimming muscle was used for all analysis [17] because muscle turnover rates are longer than those of liver and blood [18] and thus integrate diet over months. Five grams of samples were dried in the oven at 60 °C for 24 hours The samples were ground to a fine powder with mortar and pestle. The powdered samples were then divided equally into two subsamples. One of the subsamples was not acidified for the analysis of $\delta^{15}N$. Other subsamples were acidified by adding 1M of Hydrochloric acid (HCl) drop-by-drop until the bubbles were observed (the cessation of bubbling was used as criterion to determine the amount of acid to add) [19]. All the samples were left in acid for 3 h. Then, the samples were dried in the oven at 60°C until constant weight. Finally, the acidified samples were ground to a fine powder with an agate mortar and pestle again, then stored in clean desiccator until analyses. Fish samples were weighed to 0.4 to 0.5 mg for analysis using isotope ratio mass spectrometry (IRMS).

Sample Preparation

Isotopes analysis was carried out at Doping Control Centre (DCC), University Sains Malaysia (USM), using an elemental analyser Thermo Finnigan Flash EA 2000 connected to Finningan delta v-avantage plus isotope ratio mass spectrometry by a ConFlo II interface, with an analytical precision of ±0.2 ‰. The samples were analysed by combustion in quartz tubes at 950 °C. The resulting gases were carried to a second tube at 650 °C in a Helio stream. The amount of nitrogen and carbon were determined by comparing the peak areas with a standard (acetanilide). For isotopic analysis, gases passed to the mass spectrometer through an interface, that also carried out the injection of the reference N_2 and CO_2 gases.

Once in the mass spectrometer, gas molecules were ionised by electronic impact, and separated for the action of a magnetic field, depending on the relative abundances of the molecules with different isotopic compositions.

Isotopic ratios for carbon, reported as $\delta^{13}C$, were calculated as:

$$\delta^{13}C = (^{13}C/^{12}C \text{sample} / ^{13}C/^{12}C \text{standard} - 1) \ (‰) \qquad (1)$$

Isotopic ratios for nitrogen, $\delta^{15}N$, were calculated as:

$$\delta^{15}N = (^{15}N/^{14}N \text{sample} / ^{15}N/^{14}N \text{standard} - 1) \ (‰) \qquad (2)$$

Standards were VPDB (Vienna Pee Dee Belemnite) for the $\delta^{13}C$ and atmospheric nitrogen for $\delta^{15}N$.

RESULTS AND DISCUSSION

Freshwater Fishes in Temenggor and Chenderoh Lake

A total of 27 fishes representing 4 species (*Hampala macrolepidota*, *Oxygaster anomalura*, *Oreochromis niloticus* and *Cichla ocellaris*) were captured from both locations; and selected for stable isotopes analysis. H. *macrolepidota* and O. *anomalura* were the native fish species inhabiting Temenggor Lake whereas O.*niloticus* was non-native. O. *niloticus* was introduced by Trapia Malaysia Sdn. Bhd. for aquaculture and fish cultivation in the year 2007 and was rumoured by the locals to have escaped the aquaculture cages within the reservoir. In Chenderoh Lake, H. *macrolepidota* was the only native fish caught while the remaining catch consisted of C. *ocellaris*. C. *ocellaris* was introduced in the reservoir for sport fishing by anglers (Table 1).

TABLE 1. Morphological measurements for each fish species captured from December to March 2015 in Temenggor Lake and Chenderoh Lake, reported as mean ± SD, n= 3.

Location	Fish Species	Total Length (cm)	Standard Length (cm)	Weight (kg)
Temenggor Lake	*H. macrolepidota* (N)	15.5 - 34.0	12.0 - 28.0	0.07 - 0.40
	O. anomalura (N)	20.0 - 21.0	16.0 – 16.5	0.07 - 0.08
	O. Niloticus (NN)	24.0 – 31.0	20.0 – 26.0	0.04 – 0.95
Chenderoh Lake	*H. Macrolepidota* (N)	22.0 – 26.5	18.0 – 22.0	0.16 – 0.24
	C. Ocellaris (NN)	16.5 – 25.0	13.0 - 21.0	0.09 – 0.23

Where (N)= Native and (NN) = Non-native

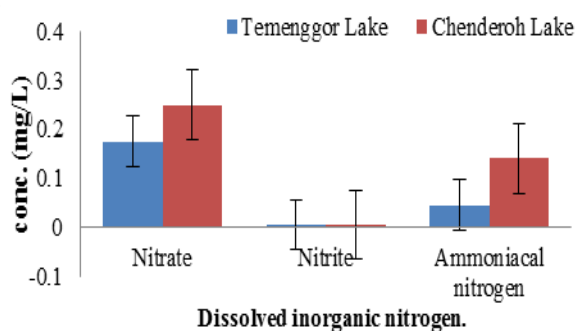### Water Quality Parameters at Temenggor Lake and Chenderoh Lake

Data obtained for water quality parameters are presented in Table 2. In Temenggor Lake, pH in the surface water

was in the range of 6.7 to 7.7 (on average 7.14 ±0.09). The pH was lower in Chenderoh Lake (6.46 – 6.64; 6.55 ±0.11). Despite the different values, the pH for both lakes were around 6.4 – 7.7, which is suitable for fish to survive [20].

Dissolved oxygen (DO)was higher in Temenggor Lake (7.4 ± 0.12) compared to DO in Chenderoh Lake (4.98 ± 0.35). The DO in lake reflects the biological productivity of the lake and may imply excessive oxygen consumed by the organisms in the waterbodies of the lake. The low DO even on the surface water can indicate anoxic stratification in the waterbodies of Chenderoh Lake. Nevertheless, in general, fish is able to survive DO of 3.0 to 7.0 mg/L in a freshwater ecosystem [21]. Conductivity and TDS were low in both lakes, indicating that low amounts of ions and dissolved solids were present in the lakes [22]. In terms of dissolved inorganic nitrogen (DIN), higher nitrate (0.18 ± 0.013 mg/L) and ammoniacal nitrogen concentrations (0.05 ± 0.005 mg/L) were measured at Temenggor Lake than Chenderoh Lake (Fig. 2). Considering DIN from surface water, this system presents a low trophic status [7]. However, further study should be carried out to investigate the effect of depth on these lakes to better clarify their trophic status.

Stable Isotopes $\delta^{13}C$ and δ ^{15}N of Fish

In Temenggor Lake, the $\delta13C$ values for H. macrolepidota, O. niloticus and O.anomalura were -25.79± 0.119, - 24.54 ± 0.142 and -30.46 ± 0.132 ‰, respectively (Fig. 3). The $\delta13C$ values in fishes reflect the carbon source in the diet of these fish species which can be derived from the primary consumer (aquatic insects, insects larvae and pupa, worms, piscivores; small fishes, fish fingerlings etc. depending on each fish species feeding habit) or primary producer (aquatic
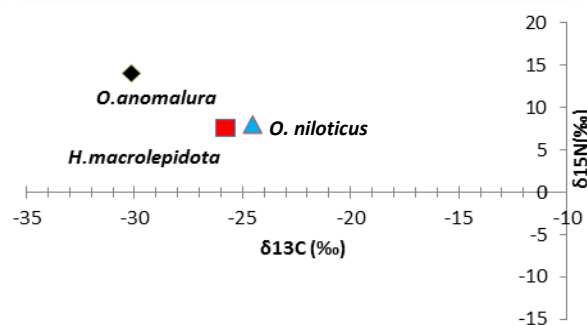
Table 2. Water Quality Parameters at Temenggor and Chenderoh Lake, reported as mean ± SD, n= 3.

Location	pH	DO (mg/L)	Conductivity (µS/cm)	TDS (mg/L)
Temenggor Lake	7.14 ± 0.09	7.14 ± 0.12	40 ± 0.38	0.027 ± 0.007
Chenderoh Lake	6.55 ± 0.11	4.98 ± 0.36	55 ± 1.14	0.032 ± 0.001

plants, macrophytes, benthic algae or phytoplankton which can be categorized as C3 or C4 plants depending on their photosynthetic pathway). Based on the literature, Garcia et al. [23] reported that C3 freshwater plants have $\delta^{13}C$ values ranging from -26.12 to -30.44 ‰, therefore we hypothesized that the carbon sources for these 3 fishes were derived from C3 plants of the reservoir. C3 plants are catalyzed by the carboxylating enzyme ribulose biphosphate carboxylase/oxygenase (rubico) in the Calvin cycle [24]. Other reported values of $\delta^{13}C$ in the literature have shown that $\delta^{13}C$ of phytoplankton and algae were in the range of -42 to -19 ‰ [25]. *O. niloticus* feeds mainly on phytoplankton or benthic algae [26] hence the $\delta^{13}C$ of *O. niloticus* may closely reflects the isotopic signatures of phytoplankton or benthic algae in the study area.

On the other hand, trophic length and trophic position of consumers is generally calculated based on $\delta^{15}N$, because of higher values of δ ^{15}N trophic fractionation. In a food chain or food web, there is normally the primary producer, primary consumer, secondary consumer and even tertiary consumer. The higher the value of δ ^{15}N for the species means that the species occupies higher trophic position in the food web. Nitrogen ($^{15}N/^{14}N$) stable isotope ratios become enriched in ^{15}N with trophic transfers, presumably due to preferential excretion of light isotopes (^{14}N) in nitrogenous waste and incorporation of heavier isotopes (^{15}N) in consumer tissues [4].

Figure 2. Dissolved inorganic nitrogen in surface water samples of Temenggor Lake and Chenderoh Lake, Perak, Malaysia, reported as mean ± SD, n= 3.

Figure 3. Stable isotope composition of freshwater fishes at Temenggor Lake ($\delta^{13}C$ vs $\delta^{15}N$)

The $\delta^{15}N$ values of *O.anomalura* was 14.029 ± 0.363 ‰ compared to 7.574 ± 0.544 and 8.013 ± 0.285 ‰ for *H.macrolepidota* and *O.niloticus*, respectively. The most enriched (most positive) $\delta^{15}N$ values of *O.anomalura,* suggests that *O.anomalura* does not occupy the same trophic level *as O.niloticus and H.macrolepidota* in Temenggor Lake (Fig.3).

The $\delta^{13}C$ values of *C. ocellaris* in Chenderoh Lake (-32.690 ± 0.675‰) and *H. macrolepidota* (-34.579 ± 0.195 ‰), falls within the range of phytoplankton as reported in Rogers [25]. Fig. 4. shows the $\delta^{13}C$ values of the two fish species captured in Chenderoh Lake.
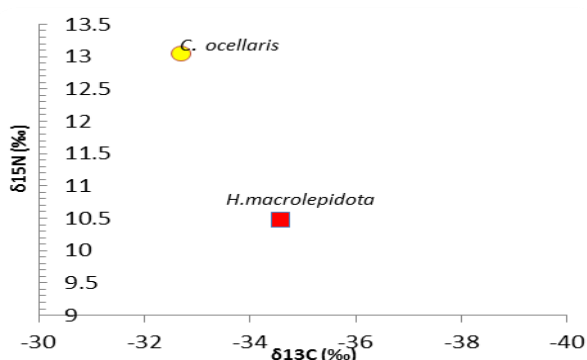
Figure 4. Scatterplots of $\delta^{13}C$ vs $\delta^{15}N$ values of freshwater fishes in Chenderoh Lake, Perak, Malaysia. H.macrolepidota = *Hampala macrolepidota* and C.ocellaris = *Cichla ocellaris*

On the other hand, *H.macrolepidota* displayed less enriched $\delta^{15}N$ (less positive) than *C. ocellaris*, implying that there is no competition amongst them for food sources. Both *H.macrolepidota and C. ocellaris* are carnivorous that only feeds on small fishes, indicating that they occupy higher trophic position in the food web. Comparison between the $\delta^{13}C$ of *H.macrolepidota* between the two lakes indicates that *H.macrolepidota* at Chenderoh Lake have more negative $\delta^{13}C$ values and more positive $\delta^{15}N$ values than *H.macrolepidota* at Temenggor Lake (Fig. 3 and Fig.4), suggesting different food sources and different trophic position of *H.macrolepidota* in the two lakes. Fish from the same species may have different isotopic composition depending on their habitat and feeding [4].

The lack of data for primary producers and primary consumers at the studied sites hindered some detailed evaluation of the proportional carbon contribution of local primary producers to fish nutrition and the exact trophic position for the fishes at Temenggor Lake and Chenderoh Lake. Fig. 5 shows the range of $\delta^{13}C$ and $\delta^{15}N$ values of different primary producers according to the literature incorporated with the $\delta^{13}C$ and $\delta^{15}N$ values from the freshwater fishes found from our study at Temenggor and Chenderoh Lake. Comparison with the literature provide some clues of the potential sources of

production in our study. However, overlapping range of the $\delta^{13}C$ for each source can occur, which makes interpretation unclear based on $\delta^{13}C$ alone [25]. Thus, $\delta^{15}N$ is used concurrently with $\delta^{13}C$ to overcome the problem.

Identification of sources of energy through identifying the producers of the ecosystem is based on $\delta^{13}C$ because the changes in $\delta^{13}C$ values at each trophic step are generally conservative. The conservative transfer of carbon isotopic compositions (within about 1‰) to the animal from the diet can be useful in tracing food webs in systems where food souces have a distinct isotopic composition [5]. The $\delta^{15}N$ values of consumers tend to be enriched by 3-4 ‰ relative to their diets because heavier ^{15}N accumulates in consumers as it moves up the food web [27,28,23,9].

Applying this rule, a conceptual food web model can be proposed for Temenggor Lake and Chenderoh Lake (Fig.6). The food web model for Temenggor Lake and Chenderoh Lake includes predictions of the $\delta^{13}C$ range to identify the sources of production and $\delta^{15}N$ values to infer trophic levels indicated by the dotted lines. The food web model shows smaller range of $\delta^{13}C$ and $\delta^{15}N$ values relative to the $\delta^{13}C$ and $\delta^{15}N$ values from the literature as shown in Fig. 5. The food web shows a typical food web in freshwater ecosystem including the characteristically $\delta^{13}C$-depleted primary production resembling C3 vegetation dominated system in the freshwater ecosystem. In terms of trophic level, we can better elucidate the specific trophic position of each organism once the actual isotopic composition of primary producers up to the primary consumers and secondary consumers are obtained from the field.
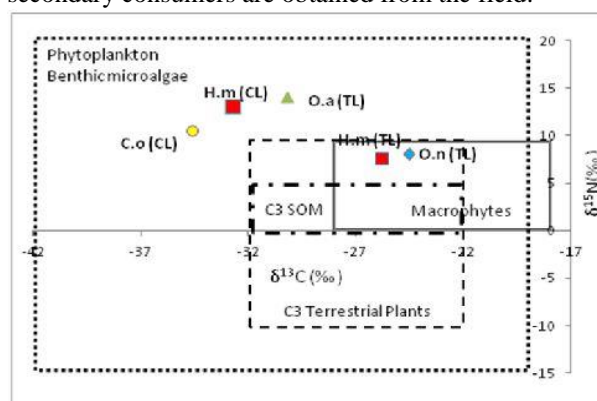
Figure 5. The $\delta^{13}C$ and $\delta^{15}N$ values of various types of primary producers based on literature survey overlaid by the $\delta^{13}C$ and $\delta^{15}N$ of freshwater fishes in our study in Temenggor Lake and Chenderoh Lake. H.m (TL) = *Hampala macrolepidota* (Temenggor Lake), O.n (TL) = Oreochromis niloticus (Temenggor Lake), O.a (TL)= *Oxygaster anomalura* (Temenggor Lake), H.m (CL) = *Hampala macrolepidota* (Chenderoh Lake), C.o (CL)= *Cichla ocellaris* (Chenderoh Lake), C3 SOM = C3 sediment organic matter.
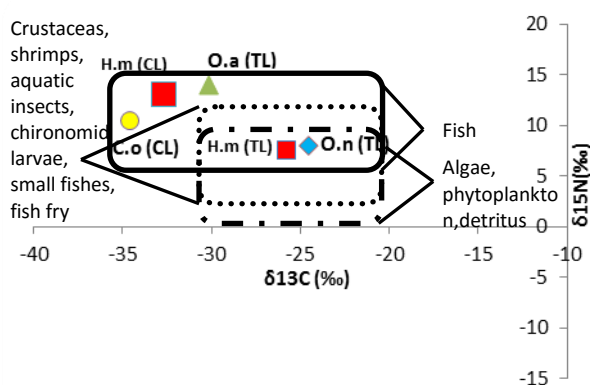
Figure 6. Conceptual food web model for Temenggor Lake and Chenderoh Lake freshwater lake ecosystem. Dotted lines represent predictions of $\delta^{13}C$ and $\delta^{15}N$ values for species which were not analyzed in our study. H.m (TL) = *Hampala macrolepidota* (Temenggor Lake), O.n (TL) = Oreochromis niloticus (Temenggor Lake), O.a (TL)= *Oxygaster anomalura* (Temenggor Lake), H.m (CL) = *Hampala macrolepidota* (Chenderoh Lake), C.o (CL)= *Cichla ocellaris* (Chenderoh Lake).

CONCLUSIONS

This study showed that carbon sources in the fishes appear to be based on C3 plants and phytoplankton assemblage in the respective lakes; Temenggor and Chenderoh Lake according to the literature. Trophic position based on the $\delta^{15}N$ values indicate that *O. anomalura* occupies the highest trophic level, presumably tertiary or higher trophic level than the other 2 fish species in Temenggor Lake. Despite its small size, *O. anomalura* is a predatory species, which might explain its trophic position higher up in the food web. In Chenderoh Lake, the $\delta^{13}C$ values not surprisingly implies the same source as Temenggor Lake since both lakes are freshwater constituents. We would expect different $\delta^{13}C$ values if we include the marine counterpart of the ecosystem as they tend to be dominated by C4 plants. In terms of $\delta^{15}N$ at Chenderoh Lake, *C. ocellaris* has a higher trophic position, confirming its predatory nature. However, to fully elucidate the food web in these lakes, further sampling of primary producers and consumers are needed to test the hypothesis of our conceptual food web model. As one of pioneer in stable isotopes analysis to determine energy flow and aquatic food web structure in tropical lakes, this work provides an important baseline information for future investigations in other tropical freshwater lakes.

ACKNOWLEDGMENTS

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چکیده

بررسی تنوع ایزوتوپ پایدار بافت‌های عضلانی گونه‌های انتخاب‌شده ماهی‌های آب شیرین از در این دریاچه‌های تمنگر و چنده رو، پراک، مالزی از دسامبر دو هزار و چهارده تا مارس دو هزار و پانزده انجام شد. هدف از این بررسی ارزیابی ایزوتوپ پایدار δ13 کربن، برای شناسایی منابع کربن و δ15 نیتروژن برای مشخص شدن منابع تغذیه‌ای ماهی‌های فوق‌الذکر. چهار نوع از گونه‌های ماهی با استفاده از روش ایزوتوپ پایدار موردبررسی قرار گرفتند که به ترتیب عبارت‌اند از: همپالا خاردار(Hampala macrolepidota) ، کپوراگزیگستر (Oxygaster anomalura)، پیکاک باس (Cichla ocellaris) وتیلاپیا نایل (Oreochromis niloticus). ایزوتوپ‌های پایدار δ13کربن و δ15نیتروژن به‌وسیله اتصال آنالیزورهای عنصری Finnigan Flash EA 2000 و Finningan DELTA V AVANTAGE همچنین طیف‌سنجی جرمی نسبت ایزوتوپ‌ها به‌وسیله دستگاه ConFlo II مورد تجزیه‌وتحلیل قرار گرفت. طبق متون علمی ایزوتوپ δ13 کربن فیتوپلانگتون C3 را گزارش می‌دهد. بر اساس ایزوتوپ پایدار δ15 نیتروژن ارزش تغذیه‌ای ماهی‌های موردبررسی نشان داد کپوراگزیگستر برخلاف جثه‌ی کوچکش به دلیل عادت غذایی گوشتخوارانه‌ی خود بیشترین ارزش غذایی را داراست، درحالی‌که گونه پیکاک باس به دلیل رفتار غارتگرانه‌ی خود به تعداد بیشتری در دریاچه چنده رویت شد. تجزیه تحلیل‌های بیشتری برای روشن شدن شبکه غذایی در دریاچه‌های گرمسیری به‌منظور مشخص شدن تولیدکنندگان و مصرف‌کنندگان اولیه باید صورت گیرد. این مطالعه یک رکورد مرجع برای انجام ایزوتوپ پایدار در شبکه غذایی اکوسیستم دریاچه‌های استوایی برای مدیریت بهتر و درک عمیق‌تر از عملکرد اکوسیستم فراهم می‌کند.