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Lipid and fatty acid fractions in *Lingula anatina* (Brachiopoda): an intertidal benthic fauna in the West Bengal-Orissa coast, India

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Comments

L. anatina, a Precambrian living fossil containing phospholipids, neutral lipids and glycolipids, especially rich in -ω series of fatty acids like eicosapentaenoic acid and docosahexaenoic acid which have pharmaceutical importance and may be linked to industry for large scale preparation.

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ABSTRACT

Objective: To record the fractional components of lipid and polyunsaturated fatty acids of *Lingula anatina* (*L. anatina*), a Precambrian intertidal benthic brachiopod, giving emphasis on -ω series group especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) alongside assessing their biotransformation within the population and mangrove-estuarine associated community.

Methods: Different biological samples after being collected from three contrasting study sites *viz.* SI, SII and SIII at Talsari (Longitude 87°5′ E to 88°5′ E and Latitude 20°30′ N to 22°2′ N) were stored at -20 °C until analyzed. Total lipids were extracted from each sample following Bligh and Dryer method. Identification and conformation of fatty acids were done by following Ackman method.

Results: On analyzing different collected samples, muscles of *L. anatina* exhibited the highest amount of total lipids (2.95%) of which 54.03% belongs to phospholipid groups. Different body parts of studied species contained appreciable and greater amount of EPA and DHA than α-linolenic acid.

Conclusions: Different collected samples exhibited variabilities in respect of total lipids and its fractional fatty acid components. The muscles of *L. anatina* showed maximum storage of lipids and fatty acids. Differential occurrences of EPA and DHA in different body parts of *L. anatina* are supposed to be due to the biotransformation process converting the α-linolenic acid from its primary food sources.

KEYWORDS

Lingula anatina, Biotransformation, ω-3 fatty acids, EPA, DHA

1. Introduction

Mangrove ecosystem, a unique, fragile, highly productive ecosystem in the sea-land interphase, is the conglomerations of plants, animals and microorganisms acclimatized in the fluctuating environment of tropical intertidal zone. Trophic interactions in mangrove estuaries are often complex, largely because of the interactions of an array of ecological parameters both living and non living pertaining to both aquatic and forest subsystems[1,2].

The fatty acids are being used as biomarkers to understand food chain and food web interactions in an estuarine ecosystem and offer a reliable method for determining important dietary information over an

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extended time span[3]. Gut content analysis only provides an understanding of the food preferences of the studied animal[4]. Biochemical analysis can help to recognize the composition and nature of fatty acids derived from their diet. The lipid components are not selectively processed during food intake and incorporation, and hence in many circumstances are integrally and markedly transferred through aquatic food webs[5]. Therefore, knowledge on fatty acid composition is supposed to focus on dietary sources of the lipids. Thus fractional composition of fatty acids is being used to follow the transfer of energy through a marine food web which in turn enables qualitative assessment of the relative trophic position of an organism[6].

Mangrove ecosystem dynamics contribute important energy transfers and have stronger trophic linkages with benthic invertebrates[2]. Lingulid macrobenthic Brachiopoda, *Lingula anatina* (*L. anatina*), a Precambrian living fossil has been reported from the intertidal mudflats of Talsari, at the confluence of Subarnarekha estuary with Bay of Bengal which contributes valuable ecological services in respect of biomass and its bioturbatory activities[7].

Besides, larvae and adult brachiopods are suspension feeders and have a lophophore as a feeding organ[8]. The vegetal and animal fractions of food of *L. anatina* are usually mixed up with a certain amount of sedimentary particles having a sizes of 2 to 3 μm and various organic detritus[9] and thereby establishes the idea that this faunal component acts as a link among different structural components of the mangrove-estuarine ecosystem.

The polyunsaturated fatty acids (PUFA) of -ω series cannot be synthesized by humans and are thus obtained through diet[10]. Although medical and nutritional importance of these fatty acid components have been established[10], very few studies have been undertaken on efficient exploitation of these natural bioactive compounds. At present, marine fishes and fish oils are the main commercial sources of PUFAs but their suitability for human consumption has been questioned from a biosafety perspective, raising the need to search for alternative sources of high quality PUFAs[11]. Furthermore, the presence of chemical contaminants (*e.g.* mercury) in fish oil can be harmful to consumers[12].

In such context, the present study on the food habits, biochemical analysis of specific lipid and fatty acid components from primary food sources to the different body parts of studied intertidal benthic animal, *L. anatina*, in a mangrove-estuarine ecosystem has been undertaken. Emphasis was also given to elucidate the spectrum of biotransformation and trophic relationships on one hand and also to recognize *L. anatina*, as the dietary source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the other.

2. Materials and methods

2.1. Collection of biological samples

The study was undertaken during July 2009 to June 2011. Mangrove leaves, detritus and *L. anatina* were collected from three contrasting study sites (namely SI, SII, and SIII) from the confluence of Subarnarekha estuary at Talsri (longitude 87°5′ E to 88°5′ E and latitude 20°30′ N to 22°2′ N) near New Digha, West Bengal, India. Both phytoplankton and zooplankton were collected by filtering 100 L of water using plankton nets having a mesh size of 0.35 μ and 0.50 μ respectively. The plankton samples, detritus, plant leaves and different body parts (separated by dissection in the laboratory) of *L. anatina* were immediately frozen and stored at -20 °C until analyzed.

2.2. Extraction of lipids

The total lipids were extracted from the flesh of the collected samples following the method of Bligh and Dyer[13] using methanol-chloroform (2:1, v/v), methanol-chloroform-water (2:1:0.8, v/v/v), and then again with the first solvent system. Samples were grounded with the solvent, filtered and residual components were extracted with the next solvent system. The process was repeated. Finally, the three extracts were pooled, diluted with water and layers generated were allowed to separate in separator funnels. The chloroform layer at the bottom was withdrawn and dried over anhydrous sodium sulphate in a freezer. The chloroform solution of lipid was evaporated under vacuum, redissolved in distilled *n*-hexane and kept at -20 °C for future use. Butylated hydroxy toluene was added at a level of 100 mg/L to the solvent as antioxidant. After dilution of the pooled extracts, a heavy white precipitate appeared at the junction of the two layers which were saved for further analysis.

2.3. Preparation of methyl esters of fatty acids

A portion of total lipid samples were dissolved in anhydrous methanol containing concentrated sulfuric acid (1%, v/v) and the mixture was refluxed for 2 h. Methanol was evaporated to a small volume and cooled. Distilled water was added to the cooled mixture and the methyl esters of fatty acids were extracted three times with aliquots of diethyl ether. The ethereal extracts were pooled and dried

over anhydrous sodium sulphate, filtered, vacuum dried, redissolved in *n*-hexane and kept for future use.

2.4. Purification of fatty acid methyl esters by thin layer chromatography (TLC)

Fatty acid methyl esters were purified by TLC using a solvent system of *n*-hexane diethyl ether (90:10, v/v). A standard methyl ester was also run on the same plate in a separate lane. The location of methyl ester bands were done by placing the TLC plate in an iodine vapour chamber. The methyl ester bands corresponding to the standard were marked and then scrapped off the plate. Methyl esters were recovered by extracting the bands in a mini column with chloroform, the later was evaporated and the methyl esters were then kept in *n*-hexane till analyzed by gas liquid chromatography (GLC).

2.5. Gas liquid chromatography

GLC of fatty acid methyl esters were done on a Chemito 1000 instrument, equipped with flame ionization detector. Quantification was done by computer using specific Clarity Lite Software.

2.6. Analysis of fatty acid methyl esters

GLC of fatty acid methyl esters was done on a BPX-70 mega bore capillary column of 30 m length and 0.53 mm i.d. obtained from SGE, Australia. Oven temperature was programmed from 150-240 °C with a rate of 8 °C/min. Initial and final times were kept isothermal for 1 min and 20 min, respectively. Injection port and detector temperatures were 250 °C and 300 °C, respectively. Nitrogen gas was used as carrier gas and its flow rate was 6.32 mL/min.

Identification of fatty acids was done by comparing their retention times with those of standards, chromatographed under identical operational conditions of GLC. Conformation of fatty acids were done by using the fatty acid methyl ester of cod liver oil fatty acids reffered by Ackman[14].

3. Results

3.1. Analysis of various body parts and primary food sources of L. anatina

Analysis of lipid contents of different parts and food sources of *L. anatina* have revealed that the muscles of *L. anatina* contained the highest amount (2.95%) of total lipids while detritus exhibited the lowest amount (0.15%) as summarized in Table 1. Analysis of muscle samples of *L. anatina* exhibited the presence of fatty acids amounting to 34.55% in neutral lipids, 11.41% in glycolipids and 54.03% in phospholipids.

Table 1

Percentage of total lipid obtained from various body parts of *L. anatina* and its primary food sources.

Sample	Amount taken (g)	Total lipid obtained (mg)	Percentage of total lipid (w/w)
Lophophore	1.08	8.5	0.79
Pedicle	3.20	11.2	0.35
Muscle	4.27	126.1	2.95
Gut content	0.56	11.7	2.09
Plankton	2.80	23.6	0.84
Mangrove leaves	16.30	28.1	0.17
Detritus	9.63	14.2	0.15

3.2. Fractional analysis of total lipids of different body parts and primary food sources of L. anatina

Fractional components of fatty acids as represented in Table 2 and Figure 1 of total lipids showed that food components of *L. anatina* included appreciable amount of α-linolenic acid (ALA). Different body parts of *L. anatina* have shown to contain different amounts of fatty acids *viz*. EPA registered the highest amount followed by DHA and then ALA. However, mangrove leaves did not reveal the occurrence of EPA and DHA. Fatty acid composition of muscles and phospholipids obtained from muscles exhibited same results as found in case of total lipids. The quantity of linoleic acid (18:2ω6) exhibited lower amount (1.2%-1.9%) in different studied samples (except mangrove leaves and detritus), but arachidonic acid (20:4ω6) recorded higher amount (2.6%-10.4%) in different studied samples (except detritus). No such components have been detected in mangrove leaves.

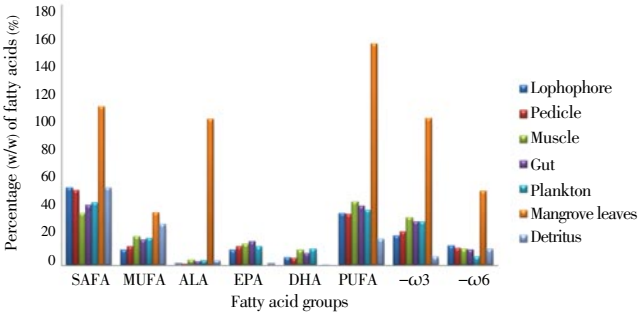

Figure 1. Fatty acid compositions of total lipids of different body parts of *L. anatina* and its primary food sources as determined by GLC of methyl esters (% w/w of each component in total fatty acids).

Table 2
Fatty acid compositions of total lipids in different body parts of *L. anatina* and its primary food sources as determined by GLC of methyl esters (% w/w of each component in total fatty acids).

Components[a]	Lophophore	Pedicle	Muscle	Gut	Plankton	Mangrove leaves	Detritus
14:0	1.40	0.90	4.50	3.10	9.00	2.10	6.50
15:0	0.90	1.20	1.30	1.30	1.30	4.20	2.40
16:0	23.00	23.40	14.70	23.20	20.40	87.00	38.20
17:0	3.30	2.90	1.80	1.90	1.10	2.10	0.60
18:0	21.30	19.80	11.70	10.10	9.50	13.50	4.40
22:0	0.70	0.80	0.50	1.00	0.30	0.20	0.40
24:0	2.70	2.80	1.20	0.60	1.30	0.40	0.70
Total SAFA	53.30	51.80	35.70	41.20	42.90	109.50	53.20
14:1					0.30		2.70
15:1		1.00			0.10	0.80	0.60
16:1	4.50	1.50	8.30	8.70	10.80	4.60	11.00
17:1	0.70	5.20	1.30	1.30	1.70	0.70	0.30
18:1ω9	4.20	3.40	8.50	7.00	5.30	29.80	13.20
22:1	0.50	0.70	1.10	0.20	0.20		0.10
24:1	0.60	0.90	0.40		0.20		
Total MUFA	10.50	12.70	19.60	17.20	18.6	35.90	27.90
16:2	0.10	0.60	0.10	0.20	0.20	0.40	1.10
17:2	2.30	0.50	0.10	0.10	2.20		
18:2ω6	1.20	1.20	1.80	1.90	1.90	49.50	9.00
18:3ω6	0.10			0.20	0.30	1.40	1.50
18:3ω3	1.00	0.70	3.50	2.50	3.10	100.70	2.70
20:3ω3	0.10	0.50	0.10	0.10	0.10		0.30
20:4ω6	10.40	9.60	7.60	5.30	2.60		0.20
20:4ω3	2.40	2.30	2.00	2.10	1.90	0.80	1.30
22:4ω6	0.50		0.80	0.80	0.60	0.18	
20:5ω3	10.10	12.70	14.40	15.80	12.50		1.20
21:5ω3	0.20		0.04		0.10		
22:5ω6	0.70	0.90	0.60	2.10	0.20		0.04
22:5ω3	1.30	1.60	2.40	1.60	1.00		0.02
22:6ω3	5.20	4.50	10.30	7.90	11.10		0.20
Total PUFA	35.60	35.10	43.74	40.60	37.80	152.98	17.56
Total-ω3	20.10	23.10	32.70	30.00	29.70	101.50	5.72
Total-ω6	12.90	11.70	10.80	10.30	5.60	51.08	10.74
PUFA/SAFA	0.67	0.67	1.23	0.98	0.88	1.39	0.33

[a]First and second figures represent carbon chain length: number of double bonds. The -ω values represent the methyl end chain from the center of double bond furthest removed from the carboxyl end. MUFA: mono unsaturated fatty acids.

Table 3
Fatty acid compositions of neutral lipids, glycolipids, and phospholipids obtained from total lipids of muscles of *L. anatina* as determined by GLC of methyl esters (% w/w of each component in total fatty acids).

Components[a]	Muscle/NL	Muscle/GL	Muscle/PL
14:0	8.50	4.90	1.00
15:0	0.30		0.40
16:0	19.90	29.50	21.50
17:0	1.50	2.60	1.80
18:0	9.30	16.80	18.00
22:0	1.70	0.80	0.40
24:0	1.90	2.20	4.50
Total SAFA	43.10	56.80	47.60
15:1		0.10	0.40
16:1	9.00	6.00	2.30
17:1	0.10	0.30	1.60
18:1ω9	7.20	5.90	3.00
22:1	0.30	0.10	0.30
24:1	0.70	0.20	0.90
Total MUFA	17.30	12.60	8.50
16:2	1.30	0.10	0.70
17:2	0.40	0.10	0.30
18:2ω6	1.20	1.30	0.90
18:3ω6	0.10	0.10	
18:3ω3	2.20	2.00	1.30
20:3ω3	0.10	0.20	0.20
20:4ω6	10.30	4.80	10.80
20:4ω3	1.30	2.00	2.60
22:4ω6	0.70	0.50	0.40
20:5ω3	11.50	9.90	13.90
21:5ω3	0.10	0.10	0.03
22:5ω6	0.90	0.60	1.20
22:5ω3	1.20	1.40	2.00
22:6ω3	6.20	3.80	7.70
Total PUFA	37.50	26.90	42.03
Total-ω3	22.60	19.40	27.73
Total-ω6	13.20	7.30	13.30
PUFA/SAFA	0.87	0.47	0.88

[a]First and second figures represent carbon chain length: number of double bonds. The -ω values represent the methyl end chain from the center of double bond furthest removed from the carboxyl end. NL: neutral lipids, GL: glycolipids, PL: phospholipids.

3.3. Fractional analysis of fatty acids of neutral lipids, glycolipids and phospholipids groups

Among three groups of fatty acids as presently demonstrated in Table 3 *viz*. neutral lipids, glycolipids and phospholipids of muscle, glycolipids showed the highest value (56.8%) of saturated fatty acids (SAFA). Mono unsaturated fatty acids of neutral lipids group registered the highest amount (17.3%) in muscles. ALA of phospholipids group exhibited the lowest amount (1.3%) among all three major groups of fatty acids. Appreciable amount of EPA (11.55%, 9.9% and 13.9%) of neutral lipids, glycolipids and phospholipids group was present in the muscles of the studied animal whereas DHA of glycolipids group revealed the presence of very little amount (3.8%).

Results on the analysis of total lipids as recorded and presented in Table 2 and Figure 1 indicated that mangrove leaves contained the highest amount of ALA whereas plankton and detritus showed the occurrence of almost equal amount of ALA. Other major fatty acid components in food sources of *L. anatina* were palmitic (16:0), stearic (18:0), oleic (18:1ω9), linoleic acid (18:2ω6) and arachidonic acids (20:4ω6). The occurrence of considerably high levels of long chain PUFA of -ω series in various organs of *L. anatina* have also been noted. Of the ω-3 PUFA recorded in this study, the PUFA/SAFA ratio have been found to vary as low as 0.33%-1.39% during present investigation (Tables 2 and 3 and Figure 1).

4. Discussion

Fatty acids have been used as qualitative markers to trace or confirm predator prey relationships in the marine

environment for more than thirty years[15]. More recently, they have also been used to identify key processes impacting the dynamics of some of the world's major ecosystems. The fatty acid trophic marker concept is based on the observation that marine primary producers lay down certain fatty acid patterns that may be transferred conservatively and hence can be recognized in primary consumers[5].

In a marine ecosystem, generally qualitative similarities are observed in the fatty acid composition of the organisms which occupy different trophic levels. The first link of the food chain *i.e.* phytoplankton are able to synthesize all the fatty acids *de novo* and composition of fatty acids changed significantly in the decomposing leaves of mangroves[16,17]. High concentration of ω-3 fatty acids which are generally considered as to be fatty acids of marine life, were found to have been mainly contributed by some phytoplanktonic species (diatoms, dinoflagellates *etc.*) to the marine ecosystem[18]. Examination on various parts of *L. anatina*, an intertidal detritivorous macrobenthic animal of the studied areas revealed the presence of appreciably high amount of 20:5ω3 fatty acids associated with other PUFA of the -ω3 series. It is thus envisaged that intake of higher ammount of the precursor acid (18:3ω3), through primary food sources and their subsequent chain elongations and desaturation processess *de novo* would lead to the formation of 3-ω unsaturated fatty acids in higher levels. Intake of these diets enriched with 3-ω acids may explain the mode of accumulation of these polyunsaturates in considerable levels in the different parts of lingulid brachiopod.

The ALA (18:3ω3), the primary precursor molecule for the -ω3 family of fatty acids[19] in animal tissues must come from diet. The principal pathways to the formation of EPA (20:5ω3) and DHA (22:6ω3) require a sequence of chain elongation and desaturation steps (Δ5 and Δ6 desaturases) with acyl-coenzyme-A esters as substrates[20,21]. The bioconversion of ALA in the present studied faunal component is supposed to occur as-

$$18{:}3\omega3 \xrightarrow{[+2C]} 20{:}3\omega3 \xrightarrow{[-2H]} 20{:}4\omega3 \xrightarrow{[-2H]} 20{:}5\omega3 \xrightarrow{[+2C]} 22{:}5\omega3 \xrightarrow{[-2H]} 22{:}6\omega3$$

(EPA) (DHA)

starting from linolenate (18:3ω3) obtained by the animal from their primary food sources which have been also supported by San Giovanni and Chew[22], and Kapoor and Patil[23].

Fatty acids are useful tools to study trophic ecology and determine food web connections, contrary to more traditional gut content analysis which provides information on dietary intake and food constituents leading to the sequestration of lipid reserves over a longer period of time[24]. The gut content analysis revealed that food contents of *L. anatina* have been found to include fragmented mangrove leaves, detritus and planktonic components which is in tune with the earlier findings conducted by Emig[9]. The presence of EPA and DHA within different body parts of *L. anatina* has strengthened the fact that these pharmaceutically important fatty acids are thought to have been derived from ALA, present in dietary food sources of *L. anatina* through biotransformation processess within the body of this rare benthic brachiopodan faunal component.

Low values of the PUFA/SAFA ratio as determined in the present research investigation are because of the presence of high levels of palmitic acid (16:0), suggesting a contribution of vegetal detritus in the diet of *L. anatina* which corroborates the findings of Reemtsma *et al*[25]. PUFAs in green algae predominantly comprised of 18:2ω6 and 18:3ω3 and these fatty acid compositions are similar to those of terrestrial (vascular) plants since they have common ancestors[26]. In the present study, an appreciable amount of 18:2ω6 and 18:3ω3 have been estimated indicating that the species under study used to consume considerable amount of green algae (phytoplankton) occurring over the surface of the soil and also from the supply of neighboring mangrove vegetations. This phenomenon further corroborates the concept as observed by Emig on the food preference of lingulid brachiopods[9]. Terrestrial organic matters can also be associated with bacteria or fungi and constitutes an attractive and energetically utilizable food sources for invertebrates[27]. In the present study, the odd branched fatty acids have been recorded as an indicator of bacterial contribution which highlights a source of food supply for *L. anatina* from decaying organic matters. Presence of arachidonic acid (20:4ω6) in different body parts of studied species further indicated that detritus serves as one part of food of *L. anatina* and this is also supported by Kelly and Scheibling[28]. During present study, it was recorded that phospholipids obtained from muscles of *L. anatina* were the major classes of lipids which form structural and functional components of cell membranes. Presence of moderate to high levels of EPA and DHA, within different body parts of studied species, derived through bioconversion of ALA from food sources indicated that they have been the good sources of EPA and DHA which are being considered as the precursors of several metabolites that are potent lipid mediators. Many investigators recognize them as to be the beneficial components for human being as in the prevention

or treatment of several diseases[29].

Present investigation has revealed that muscles of the studied animal, *L. anatina* stored major amount of all fatty acids. Presence of EPA and DHA in plankton samples indicated that *L. anatina* obtains these fatty acids from the planktons as food source. Major mangrove plant leaves of the studied area have been found to possess moderate to high amount of ALA (18:3ω3) which is the precursor of long chain PUFAs *viz*. EPA (20:5ω3) and DHA (22:6ω3). Presence of qualitatively similar type of fatty acids in *L. anatina*, inhabiting in three contrasting study sites of the Subarnarekha mangrove estuarine complex has enabled to arrive at a conclusion that the occurrence of different morphotypic forms of *L. anatina* as observed during present study belong to same genus and species. Presence of high levels of carnivorous markers of the studied species *i.e.* oleic acid (18:1ω9, derived from animal sources because of the consumption of zooplankton, animal detritus *etc*., occurred in the intertidal belts) in different body parts of *L. anatina* have indicated that they are the inhabitants of the habitat. Presence of high amount of 22:6ω3 and 20:4ω6 in different body parts of *L. anatina* has established the facts that diatoms, dinoflagellates and macroalgae constitute the basal portion of food pyramid of this complex estuarine ecosystem.

The present investigation has highlighted the mode of occurrence of lipids and its fractional components (ALA, EPA, DHA *etc*.) in different body parts (muscle, pedicle, lophophore and gut) of a Precambrian intertidal brachiopod benthic fauna, *L. anatina* inhabiting in a ecotone, the confluence of an estuary (Subarnarekha) with Bay of Bengal (longitude 87°5′ E to 88°5′ E and latitude 20°30′ N to 22°2′ N) in the North-East coast of India. Estimation of such biochemical entities has also been made from the habitat of the studied species, especially its food sources (mangrove leaves, planktons and detritus).

The variabilities in the amount of different fatty acid components in different parts and food sources of the studied species have prompted to arrive at a conclusion on the mode of biotransformation of these bioactive substances which are now being considered as the substances of pharmaceutical importance.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

L. anatina, a Precambrian living fossil collected from the Bay of Bengal, contains high quantity of phospholipids, neutral lipids and glycolipids, especially enriched with -ω series of fatty acids such as EPA and DHA. Hence, detailed investigation of fatty acid constituents of *L. anatina* is needed.

Research frontiers

L. anatina are enriched with -ω series of fatty acids such as EPA and DHA which have immense pharmaceutical importance and would be a future source of these value added products.

Related reports

Fatty acids have been recognized as qualitative markers to verify predator prey relationships in the marine environment. Recently, these are used to identify key processes impacting the dynamics of some of the world's major ecosystems.

Innovations and breakthroughs

Analysis of lipid contents of different body parts, muscle and food sources of *L. anatina* have been carried out. Fractional analysis of phospholipids, neutral lipids and glycolipids were also done.

Applications

L. anatina may be a good source of bioactive substances like -ω series of fatty acids *e.g.* EPA and DHA which have immense pharmaceutical importance and may be linked to industrial application.

Peer review

L. anatina a Precambrian living fossil containing

phospholipids, neutral lipids and glycolipids, especially rich in -ω series of fatty acids such as EPA and DHA which have pharmaceutical importance and may be linked to industry for large scale preparation.

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