Aquaculture 561 (2022) 738712

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Distribution of vitamin B12 in bivalve tissues: Investigations of larval and adult lifestages

Susanne Vogeler [a], Gary H. Wikfors [b], Xiaoxu Li [c], Justine Sauvage [a], Alyssa Joyce [a,*]

[a] Department of Marine Science, University of Gothenburg, Carl Skottbergsgata 22B, 41319 Gothenburg, Sweden
[b] NOAA Fisheries Service (NMFS) Northeast Fisheries Science Center, Milford, CT 06460, USA
[c] South Australia Research and Development Institute Aquatic Sciences Centre, 2 Hamra Ave, West Beach, SA 5024, Australia

ARTICLE INFO

Keywords:
Vitamin B_{12}
Cobalamin
Bivalves
Algae
Pacific oyster
Scallop
Clam

ABSTRACT

Shellfish, in particular bivalves, are an often-overlooked source of vitamin B_{12} (B_{12}) in the human diet although they have significantly higher tissue levels of B_{12} than other animal meat or fish sources, including all vertebrates. However, the origins and key metabolic processes involving B_{12} in bivalves remain largely unknown. In this study, we examined the distribution of B_{12} in tissues of several adult Australian bivalve species and assessed hypotheses concerning their B_{12} utilisation and principal uptake, specifically whether it is derived from diet or gut microbiome. Pacific oysters, *Crassostrea gigas*, and Goolwa cockles, *Plebidonax deltoides* ('pipis'), are both high in B_{12} (28.0–49.4 μg/100 g total per individual). Vitamin B_{12} tissue distribution, particularly in oysters, varied significantly, with higher amounts in the adductor muscle (44.0–96.7 μg/100 g), and other tissues, such as gonads, were relatively low (12.7–35.9 μg/100 g). In comparison, concentrations of B_{12} in the adductor muscle and roe of Southern Australian scallops, *Pecten fumatus*, were appreciably lower (3.4–10.8 μg/100 g). We also demonstrated that microalgal feed commonly grown in aquaculture can be supplemented directly with B_{12}, resulting in an enriched feed. However, the B_{12}-enriched diet did not transfer to a significant increase in oyster larval B_{12} concentrations, contradicting our theory that vitamin uptake through feed was a primary B_{12} source. Vitamin B_{12} concentrations across oyster larval life stages showed a significant decrease post metamorphosis, which indicates a higher utilisation of B_{12} during this life event. Our findings also provide insight into B_{12} uptake and tissue distribution in bivalve species, which can aid the aquaculture industry in promotion of bivalves as a valuable source of dietary B_{12} for human consumers, while also suggesting ways to optimise vitamin supplementation in bivalve hatchery production.

1. Introduction

Vitamin B_{12} (B_{12}), or cobalamin, is an essential vitamin for metazoan species that is required in key metabolic processes such as DNA synthesis and fatty/amino acid metabolisms, as well as playing a functional role in the nervous system (Allen et al., 2018). Deficiency in B_{12} can cause serious health conditions in humans, including pernicious anaemia, peripheral neuropathy, and other neurological complications (Allen et al., 2018; Moll and Davis, 2017). Vitamin B_{12} is absent in plants and produced de novo only by bacteria or archaea using aerobic and anaerobic pathways which no eukaryotes are known to possess (Fang et al., 2017; Rodionov et al., 2003; Roth et al., 1996). Accordingly, humans must derive required B_{12} from eating animal products or taking vitamin supplements. Dietary sources of B_{12} include animal products such as meat and dairy (Allen et al., 2018; Gille and Schmid, 2015) (recommended for adults approx. 2.4 μg per day (Institute of Medicine, 1998)). Carnivores and omnivores must acquire B_{12} from the diet; however, some herbivores have symbiotic relationships with bacteria inhabiting the digestive tract, e.g., ruminants such as cattle, that produce B_{12} in situ (Ortigues-Marty et al., 2005).

Shellfish are an underappreciated source of B_{12} in the human diet. Bivalves, such as clams and oysters, are known to have considerably higher B_{12} concentrations than livestock meats or fish, and therefore provide an excellent natural food-based source of B_{12}. Concentrations of B_{12} in bivalves range from 15 to 96 μg/100 g (Bito et al., 2018; Stabler and Allen, 2004; Tanioka et al., 2014; Watanabe et al., 2001; Yuasa et al., 2018), which is much higher than in commonly-consumed animal products, such as beef (0.7–5.2 μg/100 g), pork (0.4–2.0 μg/100 g), and

* Corresponding author.
E-mail address: alyssa.joyce@gu.se (A. Joyce).

https://doi.org/10.1016/j.aquaculture.2022.738712
Received 5 April 2022; Received in revised form 3 August 2022; Accepted 5 August 2022
Available online 8 August 2022

chicken (0.2–0.6 μg/100 g) (Gille and Schmid, 2015).

The reason for such high B_{12} concentrations in shellfish is unknown. In humans and many vertebrates, B_{12} is involved in a wide variety of metabolic functions, including in the circulatory and nervous systems, so it is surprising that shellfish have such high levels given they possess neither red blood cells nor a complex nervous system. Furthermore, it is unclear how bivalves obtain such high B_{12} concentrations because bivalves cannot produce B_{12} - only prokaryotes can. Thus, bivalves must acquire it either through their diet by feeding on microalgae, from B_{12}-rich microorganisms in their microbiome, or by assimilating dissolved B_{12}.

Approximately half of the eukaryotic microalgae are dependent upon exogenous B_{12} sources (B_{12} auxotrophs) (Tandon et al., 2017) derived primarily from heterotrophic bacteria in a symbiotic relationship (Croft et al., 2005; Croft et al., 2006; Grant et al., 2014; Kazamia et al., 2012; Wagner-Döbler et al., 2010). Algal strains, commonly grown for aquaculture diets, such as *Tetraselmis* spp., *Diacronema* (*Pavlova)* spp. and *Isochrysis/Tisochrysis* spp., contain 180 to 700 μg B_{12}/100 g dry weight, and could provide an adequate source of B_{12} for bivalves. Bivalves may also assimilate B_{12} from ingested bacteria that are part of their feed, although algae are considered their primary feed source. Alternatively, bivalves might also assimilate B_{12} from microorganisms in their gut similar to other animals, such as terrestrial ruminants (e.g., cattle, sheep) that possess a B_{12}-producing microbiome (Ortigues-Marty et al., 2005). Such relationships may be particularly important in early larval stages when bivalve larvae first acquire a gut microbiome by ingesting microorganisms from the environment (Pierce and Ward, 2018). Although it is possible that marine bivalves assimilate dissolved B_{12} directly from seawater, the extremely high concentrations in bivalves are unlikely to result primarily from this type of assimilation. Dissolved B_{12} in seawater is naturally very low, for example, ranging from 0.2 pM to 61 pM (Panzeca et al., 2009) or 3 ng/L to 605 ng/L (Okbamichael and Sañudo-Wilhelmy, 2004) in coastal areas of the Northern and Southern Atlantic Ocean.

Bivalve hatcheries aim to ensure bioavailability of key nutrients to achieve optimal rearing conditions. Hence there are practical implications for understanding which of these pathways – feed, microbiome, or dissolved vitamins – are responsible for the high concentrations of B_{12} in bivalve species. More globally, however, insights into B_{12} levels could lead to a better scientific understanding of the role of this vitamin, including its production, uptake and metabolism in humans, and could also be important for the aquaculture industry when promoting the health benefits of shellfish products.

Vitamin B_{12} deficiency is of concern in human medicine where inadequate intake and malabsorption lead to B_{12} deficiencies (Green et al., 2017; Stabler and Allen, 2004), even in countries with adequate access to animal-sourced protein. Studies have shown that B_{12} deficiency occurs in a wide range of age groups, but can be particularly important in children, women of reproductive age, and the elderly (Allen, 2008a, 2008b; Stabler and Allen, 2004). Indeed, food fortification and supplementation with synthesized B_{12} (cyanocobalamin) can reduce B_{12} deficiency; however, recent investigations have challenged the suitability of B_{12} supplements in relation to how well humans are able to assimilate specific types of synthetic B_{12}, as well as absorption limitations and genetic conditions that interfere with vitamin B_{12} uptake (Paul and Brady, 2017). For these reasons, natural sources of B_{12}, such as those that occur in shellfish, are considered preferable to supplements.

No theory has been advanced as to why bivalves have such high levels of B_{12}. In an attempt to provide more insight, we explored B_{12} distribution in different tissue types of three adult bivalve species: the Pacific oyster, the Goolwa cockle ('pipis', which are a species of clam) and the Southern Australian scallop, a common scallop species that is commercially available and widely distributed. To test the effect of B_{12} on oyster larval growth, we also experimentally varied B_{12} supply during oyster larval development by assessing whether high B_{12} diets (delivered as B_{12}-enriched microalgae) affected B_{12} concentrations in larvae. Concurrently, microalgal B_{12} concentrations in species commonly used as feed in bivalve aquaculture were examined after exposure to different concentrations of dissolved B_{12} when grown in different media. In addition, B_{12} concentrations throughout oyster larval stages were investigated in an un-replicated trial (one tank) alongside the main experiments. Information derived from these experiments provides a preliminary overview of the uptake and distribution of this key vitamin in bivalves and microalgae.

2. Materials and methods

Algal cultivation and larval experiments were carried out at the South Australian Research and Development Institute (SARDI) in Adelaide, Australia.

2.1. Vitamin B_{12} in bivalve tissues

Adult Pacific oysters, *Crassostrea gigas,* were obtained from Franklin Harbour, South Australia, including the parental generation for the spawned larvae that were utilised in subsequent larval experiments. From a local fishmonger, we purchased live Goolwa cockles, *Plebidonax deltoides* ('pipis') also from South Australia, and commercially available frozen Southern Australian scallops, *Pecten fumatus* (adductor muscle and roe) sourced from Victoria, Australia. The oyster samples were sexed prior to dissection, and the following tissue types were sampled from three males and three females: mantle (one side), gills (one side), posterior adductor muscle, gonad and digestive tract (digestive gland, stomach, midgut, intestine). The tissues of a three male Goolwa cockles (females were not available) were also dissected: foot, adductor muscles, gonads, digestive tract and remaining parts (gills, mantle and siphons). The adductor muscles and roe from three scallop individuals (simultaneous hermaphroditic species) were dissected after defrosting. Each tissue sample was rinsed in fresh tap water to remove debris, homogenised using a hand blender, and stored at −80 °C for B_{12} analysis. Weight of sampled tissues ranged from 0.399 g to 1.434 g wet weight, depending upon tissue type (S1 File). Scallops were bought frozen, and although we cannot exclude that the freezing and storage process might modify the final B_{12} concentration, single freezing cycles are not noted in prior literature to affect the final B_{12} concentrations in seawater, blood serum, or fish (Jee et al., 2014; Lees et al., 2020; Sahari et al., 2014).

2.2. Algal feed cultivation

Four species of microalgae, commonly used in bivalve hatcheries and known to be B_{12}-dependent, were used in the feeding experiments: *Tisochrysis lutea (T-iso), Diacronema (Pavlova) lutheri*, *Chaetoceros muelleri*, and *Chaetoceros calcitrans* (Croft et al., 2006; Provasoli and Carlucci, 1974). All species were grown separately in UV-treated, 1 μm-filtered seawater (38 ppt, pH 8.2) and provided with 24 h light and aeration with additional CO_2. All four algal species were cultivated in different types of media (S2 File): (1) f/2 medium (Guillard, 1975) with a final low B_{12} concentration of 3.69×10^{-10} M (1xB_{12}), (2) f/2 with a final high B_{12} concentration of 3.69×10^{-9} M (10xB_{12}), or (3) Walne medium (Walne, 1970) with a final B_{12} concentration of 7.38×10^{-9} M (20xB_{12}). Microalgae grown in f/2 media were cultured in 10 L batch cultures (carboys). Stock solutions for essential vitamins B_{12}, thiamine (B_1), and biotin (formerly vitamin H, now B_7) were prepared in 0.2 μm-filtered seawater and added to the f/2 media after the vitamin-free f/2 media was autoclaved. Microalgae grown in Walne medium were cultivated by the SARDI hatchery staff with *T. lutea, D. lutheri* and *C. muelleri* maintained separately in 50 L, semi-continuous culture bag systems. The final Walne medium for the bag system was heated to 80 °C for pasteurization and cooled before being added to the algal cultures, following standard practice for semi-continuous cultures. Batch cultures of. *C. calcitrans* grown in Walne medium were cultivated in 10 L carboys with the final

media, including vitamins, autoclaved before use (as standard for batch cultures).

Algal species grown in 1xB_{12} f/2 medium or 10xB_{12} f/2 medium, as well as in Walne medium (20xB_{12}) were sampled for B_{12} analysis from three different carboys or bags per species/medium. One additional sample of algae grown in the Walne medium (continuous bag culture) was taken for each species (*T. lutea* and *D. lutheri*). Approximately 1 L to 1.5 L per carboy or bag of each algal culture was harvested and centrifuged at 1,960 *g* for 2 min to pellet the algae. Carboys were sampled at a density of 5.1×10^6–1.5×10^7 cells/ml; whereas, bags at exponential growth phase were sampled by hatchery staff. After centrifugation, 50 mL of the original supernatant from each algal sample was collected for analysis. Algal pellets were resuspended in 50 mL seawater as an additional washing step, then centrifuged again (the second supernatant was discarded). The algal pellets and media samples were stored at −80 °C. For the B_{12} analysis, all algal samples were freeze-dried and stored at −80 °C. Dried larval pellet weights ranged from 0.041 g to 0.198 g (S1 File).

Additional media samples were collected from fresh 1xB_{12} f/2 medium, 10xB_{12} f/2 medium, autoclaved Walne medium, non-autoclaved Walne medium, and pasteurized (80 °C) Walne medium from the bag system. For each media type, three 50 mL samples were taken and frozen at −80 °C for further analysis.

2.3. Feeding experiments

Pacific oyster, *C. gigas*, larvae were derived from fourteen family lines (by strip-spawning, one male & one female/family line) of broodstock originating from Franklin Harbour in South Australia. Larvae were fed the first time 24 h post fertilisation (hpf), when larvae had reached D-shelled larval stage, and daily feedings thereafter consisted of microalgae grown either in 1xB_{12} f/2 medium, 10xB_{12} f/2 medium or Walne medium. The first 7 days post fertilisation (dpf), oyster larvae were fed with a microalgal mix consisting of *T. lutea*, *D. lutheri* and *C. calcitrans*. Both, *T. lutea* and *D. lutheri*, were given at equal ratios and the feed density was increased gradually each day from 30,000 cells/mL to 50,000 cells/mL, while *C. calcitrans* was fed at a constant volume corresponding to 20,000 cells/mL. From the eighth day onwards, *C. muelleri* was incorporated into the feed mixture when larvae reached a shell size at which this species is known to be ingested. The mixed feed then consisted of 30% *T. lutea*, 30% *D. lutheri,* and 40% *C. muelleri*. The feed density was increased to 80,000 cells/mL each day until 14 dpf and kept constant until end of the experiments, while *C. calcitrans* was fed at a constant volume corresponding to 20,000 cells/mL.

2.4. Low and high vitamin B_{12} diets during larval development

Prior the first feeding, 1 dpf (24 hpf) D-shelled larvae were placed in eight 20 L conical tanks and reared under static conditions with gently-aerated, UV-treated, 1-μm-filtered seawater (38 ppt, pH 8.2). The starting stocking density was 8–12 larvae/mL, which was gradually reduced to approx. <1 larva/mL at the end of the experiment as larvae were sampled and graded according to the hatchery's standard protocols (SARDI own protocols, similarly to description in (Wallace et al., 2008)). The experiment was carried out for 21 dpf until larvae reached the late-veliger stage. All larvae were fed daily with an algal mixture grown either in 1xB_{12} f/2 or 10xB_{12} f/2 after each tank was cleaned and refilled with fresh filtered seawater. Four individual tanks as biological replicates were maintained per algal treatment.

Over the course of the experiment, larval density and size (average of twenty randomly selected individuals) were monitored every 2–4 days and assessed under an inverted microscope (S3 File). After the experiment was terminated, larvae were washed and kept in seawater without feed for 6 h (depuration). Larvae were then settled (10,000–23,000 larvae; ~50–170 mg) and sampled as spat. All larval samples were centrifuged at 1960 *g* for 3 min to remove remaining seawater and kept at −80 °C until further analysis. Three samples were taken at the beginning of the experiment of unfed 1 dpf D-shelled larvae, centrifuged as the other samples, and stored at −80 °C. Before larval samples were sent for analysis, each sample was homogenised using a hand blender and re-frozen. The temperature of one 20 L tank was assessed continuously over 3 days from 15 dpf to 18 dpf using a temperature sensor.

2.5. Vitamin B_{12} assessment during larval development

To assess the B_{12} concentrations throughout development, a second larval experiment was conducted simultaneously using larvae from the same fertilisation event as the feeding experiment. Larvae were reared under the same conditions, but to obtain the larger required biomass for sub-sampling at different developmental stages, larvae in this treatment were instead reared in one 200 L tank under static conditions. Larvae were fed daily with an algal mixture grown in Walne medium (20× B_{12}) as outlined above. Larvae were sampled at different developmental stages, including late D-shelled larvae (3 dpf), early veliger (5 dpf), mid-veliger (10 dpf), late veliger/early pediveliger (15 dpf, before eye-spot developed), and at eyed pediveliger larvae (19 dpf). Larvae displayed the typical behaviour of competence for metamorphosis, including prominent eye-spot, sinking to the bottom, reduced velum, and crawling behaviour (extending the foot) after 19 dpf. Metamorphosis was chemically induced with (±)-epinephrine hydrochloride (Sigma-Aldrich, CAS: 329–63-5) at 10^{-4} M for 1 h (Vogeler et al., 2018), and once set, were washed and kept in seawater with algal feed. Spat (21 dpf) were collected and sampled two days post metamorphosis. Both larvae and spat were sampled in triplicate yielding three analytical-replicates and were prepared and stored as described above. Wet weights of all larval samples were recorded (S1 File). After completion of the experiment to assess the tank temperature, the 200 L tank was filled with seawater and temperature was measured continuously over two days using a temperature sensor.

2.6. Analysis of vitamin B_{12}

The B_{12} analysis was conducted by Eurofins Vitamin Testing Denmark, an accredited food lab for B_{12} testing using the *Lactobacillus leichmanii* (ATCC 7830) microbiological assay (Reference method: AOAC 952.20). Using this method, B_{12} is extracted from the samples in an autoclave using a buffer solution and then diluted in basal medium. The growth response of L. *leichmanii* to extracted B_{12} is measured turbidimetrically, which is then compared to calibration solutions with known cyanocobalamin. The limit of detection for this assay is indicated to be 0.01 μg/100 g.

2.7. Statistical analysis

Total B_{12} amounts for whole oysters and cockles were calculated as the sum of B_{12} concentrations in each tissue type corrected for its proportion of the total weight (S1 File). Whole oysters were calculated based upon sampled tissue types (excluding labial palps, heart and connective tissue, as these were not sampled). Statistical differences among B_{12} concentrations of algae, larvae, and tissue samples were conducted using the R software version 4.1.1 (R Core Team, 2021) with the *agricolae* package (de Mendiburu, 2021) and calculated using a Student's *t*-test or one-way ANOVA followed by multiple pairwise comparison using a Tukey's Honesty Significant Difference test. Significance level was chosen at alpha <0.05.

3. Results

3.1. Vitamin B_{12} in adult tissues

Vitamin B_{12} concentrations in five different tissue types — mantle,

gills, digestive tract, adductor muscle, and gonads (eggs/sperm) — of six *C. gigas* individuals, separated by sex (three males and three females), were quantified (Table 1, Fig. 1A). The B_{12} concentrations of the gill sample of male 1 and the digestive tract and sperm samples of male 3 could not be quantified by the L. *leichmanii* assay (ATCC 7830) conducted by Eurofins and are therefore missing. Overall, no significant differences were found in the average B_{12} concentrations between tissue types from male and female individuals, thus mantle, gills, digestive tract and adductor muscle samples of males and females were pooled for statistical analysis (Fig. 1A).

On average, adductor muscle samples contained the highest amount of B_{12} with 63.4 ± 7.7 μg/100 g, a significantly higher concentration compared to mantle tissue with 35.5 ± 4.7 μg/100 g, gills with 39.0 ± 2.5 μg/100 g, and female gonads (unfertilised eggs) with 26.5 ± 7.1 μg/100 g. The lowest B_{12} concentrations were recorded in male gonads (sperm) with 18.0 ± 2.0 μg/100 g and the digestive tract with 12.7 ± 2.0 μg/100 g. The total B_{12} concentration for each individual ranged from 28.0 μg/100 g to 40.7 μg/100 g with an average total B_{12} concentration of 32.8 ± 2.9 μg/100 g for the three females and one male (Male 2).

In contrast to *C. gigas*, the three male Goolwa cockle, *P. deltoides*, individuals did not vary significantly in their B_{12} concentrations across different tissue types (Fig. 1B), but overall recorded a higher average total B_{12} with 45.9 ± 1.8 μg/100 g compared to oysters (Table 1). The lowest average B_{12} concentration was measured in the foot with 36.8 ± 4.1 μg/100 g. The highest concentration was measured in the digestive tract with an average B_{12} of 53.9 ± 12.3 μg/100 g, which also displayed the largest deviation among single measurements for cockle tissues.

The scallop *P. fumatus* contained a significantly lower amount of B_{12} in the adductor muscle with 4.1 ± 0.4 μg/100 g and roe with 9.2 ± 0.9 μg/100 g compared to most other bivalve tissue types except for oyster digestive tract and gonad tissues (Table 1, Fig. 1C).

3.2. Vitamin B_{12} in algae

The four algal species all contained B_{12} when grown in 1xB_{12} f/2 (Fig. 2A): *C. muelleri* 98.0 ± 1.0 μg/100 g > *T. lutea* 71.0 ± 2.3 μg/100 g > *C. calcitrans* 49.1 ± 1.6 μg/100 g > *D. lutheri* 38.7 ± 1.2 μg/100 g. Increasing B_{12} concentrations in the media led to significant increases in B_{12} concentration in all cultured algae, with concentrations for algae grown in 10xB_{12} f/2 medium as follows: *T. lutea* 811.0 ± 29.8 μg/100 g > *D. lutheri* 249.7 ± 6.9 μg/100 g > *C. muelleri* 202.0 ± 4.4 μg/100 g > *C. calcitrans* 184.3 ± 6.4 μg/100 g. When compared to algae grown in the semi-continuous bag cultures with Walne medium, no significant increases in B_{12} concentration were observed for *D. lutheri* (604.0 ± 136.4 μg/100 g), *T. lutea* (756.5 ± 190.2 μg/100 g) or *C. muelleri* (301.0 ± 49.0 μg/100 g) from carboys with 10xB_{12} f/2. In contrast to the batch cultured algae grown in carboys, the algae grown in the bag system displayed large variance between individual samples likely resulting from variation in the growth phase of each culture (concentrations of algae in the individual bags varied). As algae in the bag bioreactors were continuous — thus in exponential phase at all times — the B_{12} concentration in each sample varied based upon the concentration of algae in the bag at the time of sampling. Analysis of the supernatant in the algal cultures, however, did not show high variance between samples for *T. lutea, D. lutheri* and *C. muelleri* (Fig. 2B). It should be noted that due to a handling error by Eurofins, two of the *T. lutea* and *D. lutheri* media samples could not be analysed using the microbiological assay. In general, media samples for each algal species displayed a similar pattern in B_{12} concentrations, with lower concentrations for 1xB_{12} f/2 medium and significantly higher concentrations in 10xB_{12} f/2 medium, except for *C. muelleri*. Interestingly, the media in the three bag-grown algal species (Walne) — *T. lutea, D. lutheri* and *C. muelleri* — displayed significantly higher B_{12} concentrations than 10xB_{12} f/2 samples, suggesting that the continuous inflow of media in the hatchery bag system led to microalgal accumulation of B_{12}. Alternatively, it is possible that a general higher starting concentration was delivered in the growth media supplied to the bags as a result of a preference by the hatchery staff for high levels of vitamins and trace minerals in the stock solutions (to counteract unknown effects of pasteurization). This was confirmed by analysing Walne medium in unused bags (Fig. 2C), which showed significantly higher B_{12} concentrations compared to the unautoclaved Walne medium that was prepared following a standard protocol (S2 File).

Compared to the other three algal species, *C. calcitrans*, which was cultured exclusively in carboys (batch cultures) rather than the continuous culture bags, displayed a divergent pattern of B_{12} distribution. Although the B_{12} concentrations in algae from 10xB_{12} f/2 were significantly higher than from 1xB_{12} f/2, the cultures grown in Walne medium were significantly lower with 69.2 ± 1.6 μg/100 g (Fig. 2A). This was also observed in the media supernatant for *C. calcitrans* cultures (Fig. 2B). The carboys with Walne enrichment were autoclaved after vitamins were added, a practice that is often followed in hatcheries. A

Table 1
Vitamin B_{12} concentration in female and male Pacific oyster (*Crassostrea gigas*) individuals, male Goolwa cockles ('pipis'; *Plebidonax deltoides*) and Southern Australian scallops (*Pecten fumatus*) individuals ([†] hermaphrodites). *: total B_{12} per individuals as sum of the concentrations of each tissue corrected for wet weight of tissue to total weight; SE: standard error.

Tissue	Female 1	Female 2	Female 3	Male 1	Male 2	Male 3	Average ± SE
Crassostrea gigas							
Mantle	47.4	19.7	36.7	48.2	25.7	35.0	35.5 ± 4.7
Gills	37.5	30.4	44.5	n.a.	43.7	38.8	39.0 ± 2.6
Digestive tract	14.3	13.0	9.7	19.0	7.6	n.a.	12.7 ± 2.0
Adductor muscle	61.9	58.2	70.8	96.7	48.5	44.0	63.4 ± 7.7
Gonad (eggs)	35.9	31.0	12.7				26.5 ± 7.1
Gonad (sperm)				20.0	16.0	n.a.	18.0 ± 2.0
Total* (μg/100 g)	40.7	28.0	33.8	n.a.	28.6	n.a.	32.8 ± 2.9
Plebidonax deltoides							
Foot				44.6	35.1	30.8	36.8 ± 4.1
Remaining parts				44.9	50.9	53.2	49.7 ± 2.5
Digestive tract				78.4	39.0	44.4	53.9 ± 12.3
Adductor muscle				43.6	44.3	42.7	43.5 ± 0.5
Gonad (sperm)				44.7	42.5	47.0	44.7 ± 1.3
Total* (μg/100 g)				49.4	43.7	44.7	45.9 ± 1.8
Pecten fumatus[†]							
Adductor muscle	4.7	4.3	3.4				4.1 ± 0.4
Roe (eggs)	10.8	9.2	7.6				9.2 ± 0.9

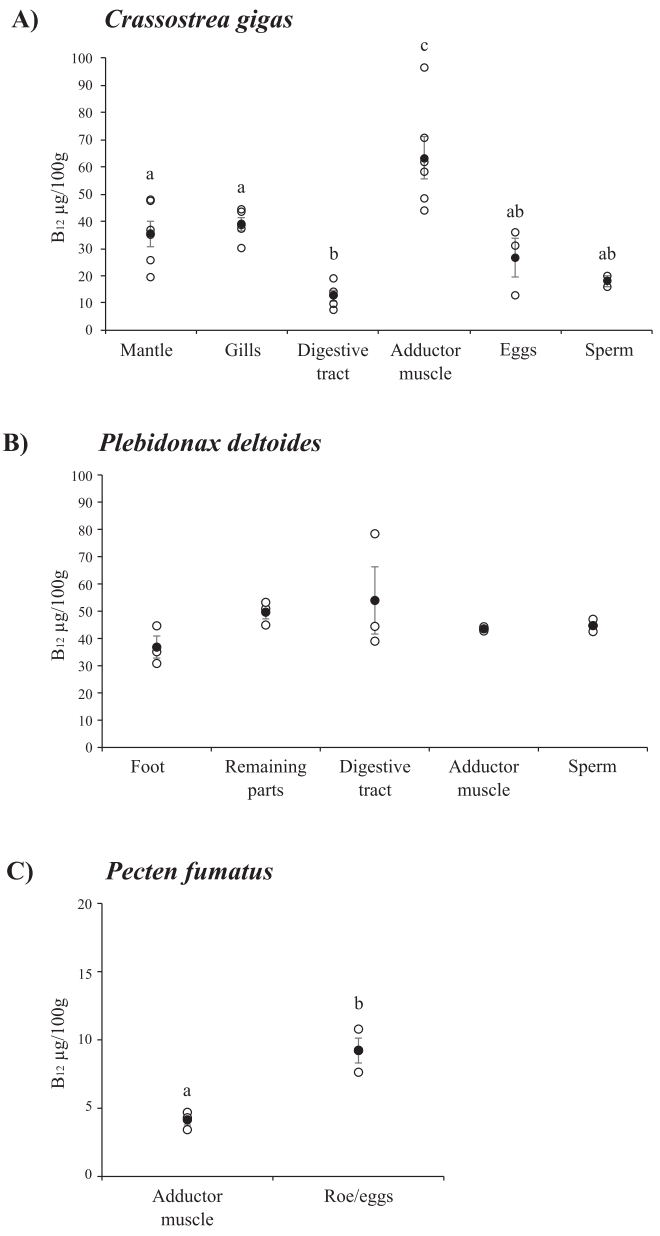

Fig. 1. Vitamin B_{12} (µg/100 g) concentrations in different tissue types of bivalve species.
A) Pacific oyster, *Crassostrea gigas;* B) Goolwa cockles, *Plebidonax deltoides*; C) and Southern Australian scallop, *Pecten fumatus*. Average B_{12} concentration (● filled circles) and individual (○ open circles) measurements per tissues type for the three bivalve species. Male and female oyster tissues were pooled (except gonads) due to the fact we observed no significant difference based on sex. Error bars for average concentration: standard error; letters above show significant differences ($p < 0.05$).

direct comparison of autoclaved and non-autoclaved fresh Walne media showed a significant decrease in final B_{12} concentrations in autoclaved media (Fig. 2C).

3.3. Vitamin B_{12} in oyster larvae

Vitamin B_{12} concentrations and growth were assessed in oyster larvae after feeding with algae grown in media with different B_{12} concentrations. In general, larvae reared in tanks of 20 L volume fed with algae grown in f/2 ($1xB_{12}$ & $10xB_{12}$) grew to late veliger stage at 21 dpf, but did not reach eyed pediveliger stage at termination of the experiment (no foot visible). Significant differences in average size between the two diets were not observed with an average size of 229.8 ± 2.3 µm for larvae fed with $1xB_{12}$ f/2 algae, and 217.6 ± 2.8 µm for larvae fed with $10xB_{12}$ f/2 algae after 20 dpf (Fig. 3A). The 20 L tank measured had an average temperature of 23.30 ± 0.03 °C ranging from 22.6 °C at night to 25.9 °C during the day.

Overall, different B_{12} concentrations in algal feed did also not significantly change the B_{12} concentrations of late veliger oyster larvae (Fig. 3B). Larvae fed with algae grown in f/2 with different B_{12} concentrations did vary significantly in their final average concentrations after 21 dpf with $1xB_{12}$ f/2 larvae containing 63.2 ± 5.8 µg/100 g and $10xB_{12}$ f/2 larvae containing 88.8 ± 20.30 µg/100 g, although the observed trend indicates higher B_{12} levels in the larvae fed with high B_{12} algal feeds. Furthermore, B_{12} concentrations of late veliger larvae independent of diet did not differ significantly when compared to unfed larvae at 1 dpf with an average B_{12} concentration of 50.0 ± 3.0 µg/100 g.

Data on B_{12} concentrations across different larval developmental stages fed with algae grown in Walne medium from the single 200 L tank shows that B_{12} concentrations did not significantly change for larvae fed with algae grown in Walne medium (Fig. 3C). Larvae in this tank reached eyed pediveliger stage and grew significantly faster than larvae in the smaller tanks (Fig. 3A). They were close to metamorphosis after 19 dpf, with an average size of 317.0 ± 2.3 µm. However, after metamorphosis mean B_{12} concentration decreased significantly in two-day old spat (21 dpf) to 28.1 ± 2.3 µg/100 g (Fig. 3C) compared to all larvae stages. In comparison with the larvae fed with the two f/2 diets, the larvae fed algae grown in Walne medium did not show a significant difference in B_{12} levels, despite receiving feed with a much higher B_{12} concentration. There was no difference in the 200 L tank samples either in late veliger larvae at 15 dpf with 54.4 ± 1.33 µg/100 g, or eyed pediveliger larvae at 19 dpf with 51.8 ± 3.83 µg/100 g B_{12} (Fig. 3B). The temperature in the 200 L tank varied from 24.40 °C to 26.15 °C, with an average of 25.38 ± 0.03 °C.

4. Discussion

The present study confirms, and adds to existing knowledge that shellfish, such as bivalves, are an excellent natural source of bioavailable B_{12} (Bito et al., 2018; Maxwell, 1952; Ueta et al., 2011; Ueta et al., 2010; Watanabe et al., 2001; Yuasa et al., 2018). Bivalves have higher B_{12} concentrations than standard meat and dairy products (Gille and Schmid, 2015) and greater bioavailable B_{12} than other marine species analysed, such as crustaceans, abalone and herbivorous snails (Maxwell, 1952; Tanioka et al., 2014). Most prior studies have focused on vitamin concentrations in whole animals, with almost no information provided on how B_{12} is distributed in the different tissues of bivalve species.

The present study found that Pacific oysters had comparable B_{12} concentrations (28.0–40.7 µg/100 g) to what was previously reported in the literature (15.1–46.3 µg/100 g) (Bito et al., 2018; Watanabe et al., 2001; Yuasa et al., 2018). The results also indicate that most B_{12} in Pacific oysters is stored in the adductor muscle, accounting for approximately 31–35% of total soft-tissue B_{12} (S1 File); whereas the lowest concentration of B_{12} was found in the digestive tract. This finding was surprising given that the digestive tract could be presumed to be the organ for intake and absorption of B_{12}. Most vertebrates store B_{12} in the liver; in invertebrates, the digestive gland/hepatopancreas is the analogue to the vertebrate liver. In shrimp, for instance, B_{12} is assumed to be stored in the hepatopancreas (Shiau and Lung, 1993). Thus, it would have been expected to find high levels of B_{12} in the digestive organs of oysters, given that B_{12} could have been sourced from food, or produced by microbial sources in the gut. These findings are further confounded by the fact that oysters seem to be unique in their storage of B_{12} in the adductor muscle, as the two other bivalve species analysed – Goolwa cockles and scallops – did not exhibit a similar pattern. The Goolwa cockle, higher in B_{12} overall, did not store the majority of its B_{12}

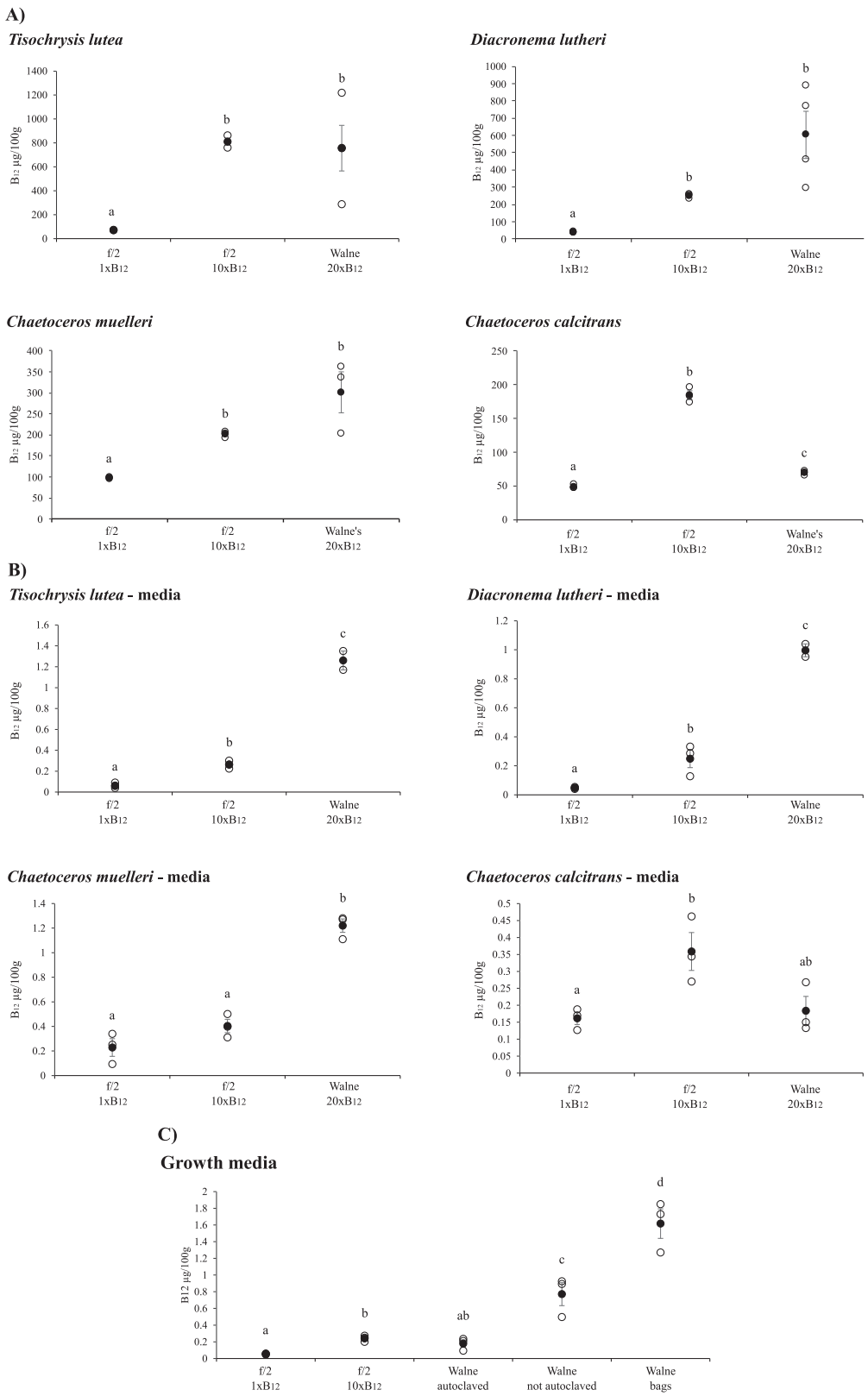

Fig. 2. Vitamin B_{12} (µg/100 g) concentrations of microalgal species.
A) Average B_{12} concentrations in the four microalgal species (*Tisochrysis lutea, Diacronema (Pavlova) lutheri, Chaetoceros muelleri* and *Chaetoceros calcitrans;* freeze-dried) grown in $1xB_{12}$ f/2, $10xB_{12}$ f/2 and Walne media ($20xB_{12}$) and B) of the media supernatant of the four algal species. Algae grown in $1xB_{12}$ f/2 and $10xB_{12}$ f/2 media as well as *C. calcitrans* Walne medium were cultivated in 10 L carboys, while the remaining algal species grown in Walne medium were cultivated in a bag system. C) B_{12} concentration of fresh growth media for each medium type. ○ Open circles: individual measurements, ● filled circles: average of all measurements for this sample point including standard error (error bars), letters above show significant differences ($p < 0.05$).

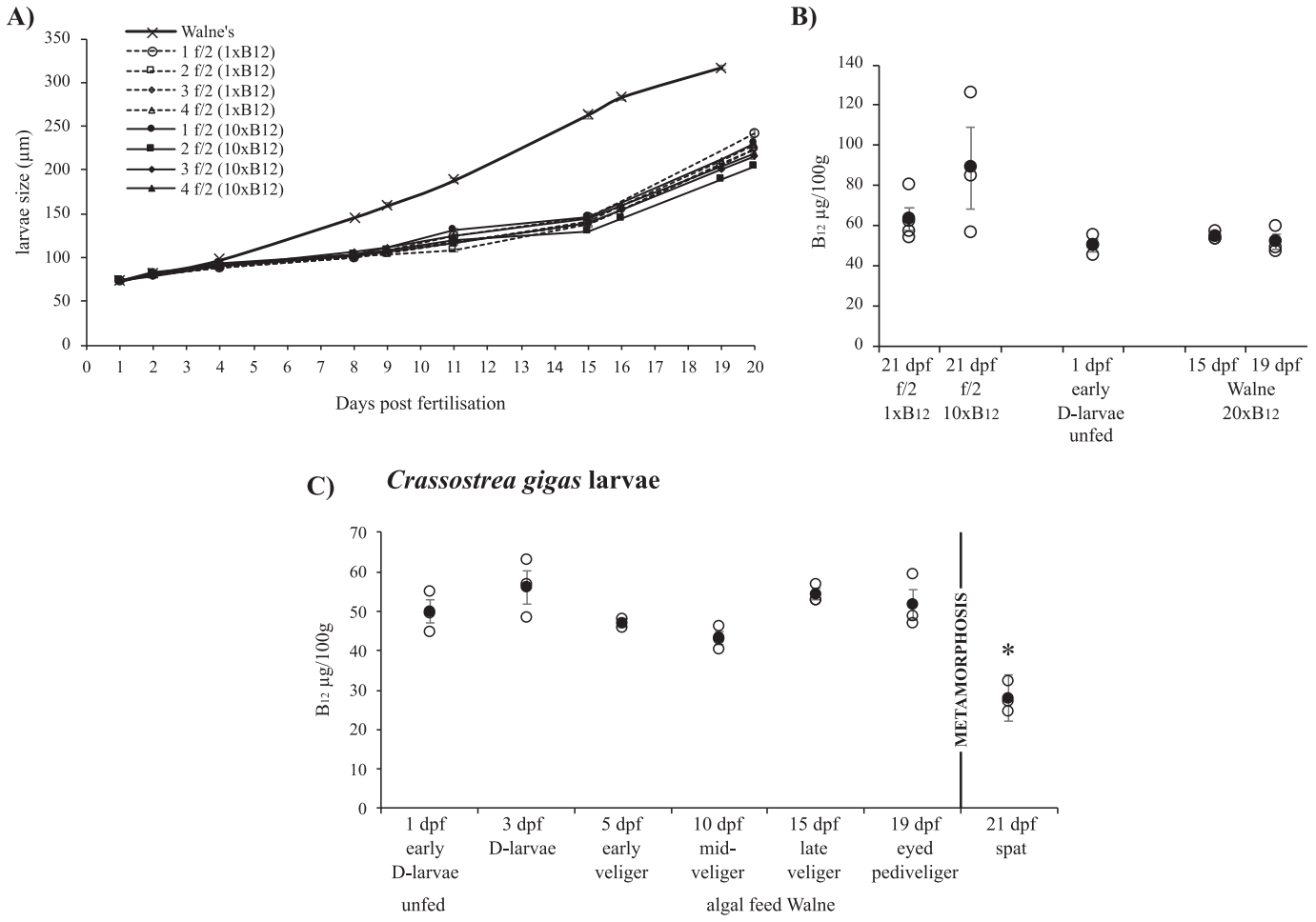

Fig. 3. Vitamin B_{12} (µg/100 g) concentrations of Pacific oyster, *Crassostrea gigas*, larvae.
A) Average larval size (µm) during development from 1 day post fertilisation (dpf) until end of experiments with final size assessment on 19 dpf for larvae in one 200 L tank fed with algae grown in Walne medium and on 20 dpf for larvae in each four separate 20 L tanks fed with algae grown in f/2 media ($1xB_{12}$ & $10xB_{12}$). B) B_{12} concentrations of 21 dpf larvae fed with algal mix grown in f/2 medium, unfed 1 dpf D-shelled larvae and 15 dpf and 19 dpf larvae fed with algal mixture grown in Walne medium. C) Average B_{12} concentrations at different larval live stages. Early D-shelled larvae (1 dpf) were assessed prior first feeding and thereafter fed with algal mixtures of algae grown in Walne medium. ○ open circles: individual measurements, ● filled circles: average of all measurements for this sample point including standard error (error bars), *: significant difference ($p < 0.05$) of spat to all larval stages.

exclusively in the adductor muscle, rather all tissues contained about the same B_{12} concentration. The higher fluctuations between digestive tract measurements could be a consequence of algal remains in the digestive tract given that algae contain large amounts of B_{12}. Interestingly, based upon the percentage of B_{12} amounts per body part, most of the cockles' B_{12} (34–44%) is stored in the remaining soft tissue such as siphons, gills and mantle tissue. Further research is needed to specify if any of these remaining body parts is particularly high in B_{12}. Other molluscan species, such as gastropods and edible marine snails, display much higher concentrations in their visceral tissue than in the edible muscle tissue (Tanioka et al., 2014; Teng et al., 2015).

Vitamin B_{12} concentration in the scallop adductor muscle was significantly lower than that in oysters, with an average of 4.1 ± 0.4 µg/100 g. Previous work has reported a B_{12} concentration of 13.4 ± 1.0 µg/100 g for the fresh whole body of the scallop species *Mizuhopecten yessoensis* (Watanabe et al., 2001) and substantially lower concentrations in adductor muscle at 1.1 ± 0.2 µg/100 g (Tanioka et al., 2014). Similar methods for B_{12} detection were utilised in those studies, suggesting that the adductor muscle is also not the key tissue for B_{12} storage in scallops.

The function of high B_{12} concentrations in bivalves and other molluscan species is speculative. In terrestrial animals, as well as fish, B_{12} is required as a cofactor for two B_{12}-dependent enzymes, methylmalonyl-CoA mutase (MCM) for mitochondrial conversion of methylmalonyl-CoA to succinyl-CoA, and methionine synthase (MetH), that catalyses the re-methylation of homocysteine to methionine, an essential amino acid (for review see (Froese et al., 2019)). We identified predicted homologues of both B_{12}-dependent enzymes in the genomes of the Pacific oyster *C. gigas* (protein GenBank ID: MCM (XP_034309642) & MetH (XP_034325685)) and the scallop *P. maximus* (protein GenBank ID: MCM (XP_033762147) & MetH (XP_033734273)). However, it is unknown if B_{12} in molluscs functions similarly to key functions in vertebrates, where it is known to be involved in the health of nervous tissue, particularly myelin synthesis (Calderón-Ospina and Nava-Mesa, 2020) and erythropoiesis (Koury and Ponka, 2004). In marine bivalves, and invertebrates generally, B_{12} might fulfil other functions potentially involved in the immune system. Non-specific immune responses, including haemocyte counts, improved at optimal B_{12} concentrations in the juvenile Chinese mitten crab, *Eriocheir sinensis* (Wei et al., 2014). Many *Vibrio* spp. are thought to be B_{12} scavengers (Agarwal et al., 2019); thus a possible mechanism to remove B_{12} from the gut or gills may also help control bacterial populations by scavenging free B_{12}. Vitamin B_{12} is known to have a critical role as an antioxidant, and thus may also aid in osmotic regulation, a particularly important function for an intertidal species facing strong salinity fluctuations and long periods when the

shell must remain closed during tidal fluctuations. The high levels of B_{12} in bivalve shellfish could therefore be hypothesised to play a role in oxidative stress responses by reducing homocysteine levels (Mikkelsen and Apostolopoulos, 2019; Van De Lagemaat et al., 2019). Although plants do not generally require B_{12}, since they contain an alternative B_{12}-independent form of methionine synthase (MetE) (Eichel et al., 1995), application of additional bioavailable B_{12} has been demonstrated to increase phenolic compounds that are able to protect plants and germinating seeds against oxidative stress induced by salinity (Keshavarz and Moghadam, 2017). Vitamin B_{12} deficiency also leads to oxidative stress and memory impairment in annelids (Bito et al., 2017). High demand for MetH-catalysed methionine production might also be a unique trait of shell producing animals, given that some bivalves such as pearl oysters contain unique proteins for biocalcification of shells that are remarkably rich in methionine (Kintsu et al., 2020; Marie et al., 2012; Suzuki et al., 2019). Besides the unknown functions of B_{12} in bivalves, where this crucial vitamin is derived – whether from algal feed or microbiome – has still not been confirmed.

Microalgal species, such as *T. lutea*, *D. lutheri*, *C. muelleri* and *C. calcitrans* commonly used in hatcheries as larval feed, are B_{12}-dependent (Croft et al., 2006; Provasoli and Carlucci, 1974) and thus high-density algal cultures are always supplemented with B_{12} in their growth media. Haptophytes such as *T. lutea* contain only a MetH homologue (Nef et al., 2019), but *Chaetoceros* spp. also contain MetH with some species additionally expressing MetE (Ellis et al., 2017). *D. lutheri,* in addition to MetH, may be able to remodel pseudo-vitamin B_{12} (Helliwell et al., 2016), a non-bioavailable form of B_{12} for most eukaryotes. In the absence of bacteria that produce B_{12} – such as occurs in axenic algal cultures – microalgae can assimilate B_{12} from the growth media (Carlucci and Silbernagel, 1969; Provasoli et al., 1957). Our results show that B_{12} concentrations in these four microalgal species were significantly increased by providing elevated B_{12} in the growth media. Algae grown in the bag system showed a larger variability in B_{12} concentrations compared to carboy-grown algal cultures that we cannot fully explain, given we did not see similar variability in the corresponding media supernatants. The bag systems were continuously provided with enrichments and were also being harvested continually as feed for hatchery production. Consequently, not all sampled bags would have been at similar densities, and drip rates of media and water may have varied slightly, thus influencing the final B_{12} concentrations based upon different levels of media and density of cultures. Whether or not there is differential B_{12} uptake during different growth phases is not known, although B_{12} has been shown to be variable in batch cultures in which uptake is highest in the exponential - rather than lag or static - growth phase (Nef et al., 2019). Levels of B_{12} observed in the diatom *C. calcitrans*, however, which was cultured only in carboys (it does not grow well in hatchery bag systems), demonstrated an important principle related to vitamins in growth media. The B_{12} concentrations in Walne growth medium decreased significantly after autoclaving, wherein the high temperature destroys vitamins such as B_{12} which also resulted in decreased B_{12} concentrations in the algal cultures. Although not all hatcheries autoclave vitamin solutions, this practice is relatively common and is worthy of note given the sharp drop in B_{12} after autoclaving. The amounts stipulated in media formulations were designed to be in excess for this reason; however, it was not clear if this could nonetheless reduce the growth rate of *C. calcitrans,* or have subsequent effects upon larval rearing. Previous research has shown that increased B_{12} in f/2 growth medium does not result in increased algal growth rate, as long as needed B_{12} concentrations are being met (Krichnavaruk et al., 2005).

When provided with B_{12}–rich diets, Pacific oyster larvae did not deplete or significantly bioaccumulate B_{12} throughout larval development. They appeared to maintain similar levels of B_{12} compared to the early D-shelled stage prior to first feeding, thus suggesting that larvae already start out with high B_{12} concentrations derived from non-algal sources. Our hypothesis that differences in B_{12} concentrations of algal feed might be reflected in B_{12} levels in larvae, which would have supported a theory of uptake from dietary sources, was not confirmed by the results reported here. Indeed, the B_{12} provided appears to be adequate in all treatments. Although a weak trend toward higher B_{12} concentrations in 10xB_{12} f/2 was seen, the vitamin concentration did not significantly differ from either 15 dpf larvae (closest in size to f/2 larvae) or 19 dpf larvae (closest in age) fed with Walne-grown algae that contained the highest B_{12} concentrations of all treatments, including the B_{12} enriched f/2 medium. These results do not provide convincing evidence that larvae obtain B_{12} primarily from microalgal feed. Given that limited information is available on larval dietary B_{12} requirements, any potential beneficial effect of B_{12} uptake through diet might have been inconsequential, because even the lowest B_{12} concentrations tested met those minimal requirements. A further decrease in the B_{12} concentration in algal feed could shed additional light on this question.

Higher B_{12} concentrations in the diet did not significantly increase larval growth rates, as seen for the two f/2 algal diets, suggesting that enriching algal feeds with B_{12} alone does not provide an advantage to the aquaculture industry in relation to improving larval growth. Presuming that a minimum level of B_{12} is available, additional B_{12} did not appear to provide any visible benefit, although we did not perform stress or immune challenges to determine if there may, in fact, be benefits for larval survival under adverse conditions. A significant increase in larval growth rate was seen for larvae fed with Walne-grown algae; however, this observation needs to be interpreted with caution firstly because of a lack of biological replication (these larvae were an in-kind gift from the hatchery thus were not replicated as part of the treatments), and secondly because of a potential effect of temperature. Acceleration of larval development may be a result of the more stable temperature in the 200 L tank compared to the fluctuating lower temperatures in the smaller 20 L tanks; all other factors being equal (density, and approximate feed availability), temperature was in this case likely a more important predictor of growth rates in bivalves than our experimental treatments (Helm et al., 2004). Walne and f/2 seawater enrichments not only vary in B_{12} concentrations, but also in levels of other essential nutrients such as nitrogen. Whether or not the Walne medium improved the quality of the algal diet, and eventually benefited larval development, was not tested in this study.

A significant decrease in B_{12} concentration was recorded in spat after metamorphosis, suggesting that stored B_{12} in larvae was utilised to aid in metamorphosis, but the importance of this vitamin to metabolic function during this key life event is unknown. However, based on the known anti-oxidant properties of B_{12}, as well its important role in neurogenesis in other animals, the depletion of B_{12} reserves during metamorphosis is not surprising. Indeed, metamorphosis appears to be a key life stage wherein B_{12} is important, thus suggesting that further work on larval B_{12} reserves in relation to settlement could be a valuable approach with regard to hatchery productivity. For instance, feeding spat for a short duration with a high B_{12} diet after metamorphosis did not appear replenish the B_{12} concentrations to levels observed before metamorphosis, but it is difficult to draw conclusions given our experiments were terminated shortly after metamorphosis. A more in-depth assessment of larvae and spat before, during, and after metamorphosis with different B_{12}-containing algal diets might provide further insights into B_{12} sources for larvae and spat. It may also shed further light on difficulties of achieving metamorphosis in several key commercial species where levels may be deficient. However, we cannot exclude entirely that the decrease in vitamin concentration observed in spat is a consequence of the higher shell to soft-tissue mass ratio relative to larvae in our samples. Given that the B_{12} analysis methods are accurate primarily on soft tissue, we cannot exclude this interpretation, and believe further experiments should be conducted in this regard.

The source of B_{12} in bivalve molluscs, whether from the algal diet or microbial sources, remains elusive. Prior work has shown that the levels of B_{12} in various clam/cockle species (prior unpublished work on species outside of Australia) is exceedingly high: even higher than reported in

prior literature on other bivalves, such as oysters and mussels. Although our results provide some evidence that B_{12} can be sourced from enrichments added to microalgal growth media, several other possible sources of B_{12} are worthy of consideration. For pre-feeding larval stages, nutrients that include essential vitamins might be supplied by maternal egg reserves, as previously observed in bivalves and other animals (Na et al., 2018; Seguineau et al., 2001a; Seguineau et al., 2001b; Wilson, 1997). Our data, however, indicate that B_{12} concentrations in unfertilised eggs are significantly lower than those of unfed D-shelled larvae, suggesting that another source of B_{12} than their egg reserves is also available to larvae. The trochophore life stage, a free-swimming larval stage prior to shelled D-shaped bivalve larvae, has basic structures such as a mouth, digestive mass (anlagen of the stomach) and intestine, and trochophores ingest particles in surrounding water by filter feeding – thus possibly ingesting symbiotic bacteria that could provide B_{12} to the host. Gut microbiome analysis of adult bivalves and whole *Crassostrea* spp. veliger larvae have revealed that the majority of microorganisms are *Proteobacteria (*up to ~95% in larvae) and *Cyanobacteria* ((Pierce and Ward, 2018) and the references in (Asmani et al., 2016; Stevick et al., 2019)). *Cyanobacteria* are known to produce pseudo-vitamin B_{12}, the non-bioavailable form of B_{12} for most eukaryotes (Helliwell et al., 2016; Miyamoto et al., 2006; Watanabe et al., 1999), and are therefore not likely to contribute to bivalve B_{12} uptake. However, approximately 45% of the *Proteobacteria* are predicted to be B_{12} producers (Shelton et al., 2018), in particular *α-Proteobacteria* and *γ-Proteobacteria* in marine environments (Doxey et al., 2015; Heal et al., 2017; Sañudo-Wilhelmy et al., 2014; Vitreschak et al., 2003), and those are also abundant in the microbiota of late *C. gigas* larvae (up to ~65% in 16 dpf larvae) (Asmani et al., 2016). Thus, various prospective B_{12}-producing bacteria are potentially being consumed and digested, or are colonizing the gut of bivalves, thereby providing a stable source of B_{12}. This is partially supported by a study of four gastropod species that found of 270 bacterial strains isolated from the gastrointestinal tract, 87% were B_{12}-producing bacteria (Sugita et al., 1991). These authors, however, concluded that only 6% of these bacteria showed high productivity compared to bacteria in surrounding seawater, and none of them wwas a dominant species. Nevertheless, recent studies have shown that microbiomes of oysters can vary throughout larval life (Laroche et al., 2018) as well as under stress conditions (high pH) (Vignier et al., 2021), between hatcheries (Arfken et al., 2021), and at rearing locations (Trabal Fernández et al., 2014). However, larvae and adults usually contain a small core microbiome (see (Pierce and Ward, 2018) and the references in (King et al., 2012; Trabal Fernández et al., 2014)), which could potentially hold the answer to B_{12}-producing bacterial symbionts. Further research on oyster larvae and adults is needed to shed light on this, including research into the microbiota of trochophore larvae with a special focus on the abundance of B_{12}-producing bacterial species. Identifying these bacteria in bivalve microbiomes will also aid our understanding of the potential effects of ambient bacterial composition on concentrations in the bivalve gut and gills. Water microbiomes across hatcheries and rearing locations vary and subsequently shape the microbiome of the bivalves grown in these waters (Arfken et al., 2021; Trabal Fernández et al., 2014). If bacteria are the main B_{12} suppliers to bivalve hosts, differences in environmental bacterial composition will influence B_{12} concentrations within and between species, and among habitats. The microbiomes of fertilised eggs could also provide some insight into potential vertical or/and horizontal transfer of bacteria from egg to embryo, as previously reported in fish species (Llewellyn et al., 2014). Furthermore, detection and localisation of cobalamin binding intrinsic factor, a glycoprotein required for uptake of B_{12} that is normally present in the gut of animals, is predicted in *C. gigas* (GenBank: LOC105331555); its presence could shed further light on where and how the majority of B_{12} uptake occurs in both larvae and adults.

Finally, in addition to the gut, other potential microbiomes could be considered as sources of B_{12} in juvenile (post metamorphosis) and adult bivalves. Gills host a variety of microorganisms, and previous research has shown that oyster gills can have higher bacterial diversity than the digestive gland, including a large variety of *α-* and *γ-Proteobacteria* (Hernández-Zárate and Olmos-Soto, 2006; Wegner et al., 2013). Symbiotic relationships between gill bacteria and their bivalve hosts have been well studied in the context of chemosynthetic symbioses (Dubilier et al., 2008; Roeselers and Newton, 2012) as well as the supply of digestive enzymes for celluloses and lignin (O'Connor et al., 2014; Stravoravdis et al., 2021). In our study, B_{12} concentrations in *C. gigas* gills were relatively high, as well as high in the 'remaining parts' of Goolwa cockles that included gills. This could therefore indicate potential symbioses of gill tissues with B_{12}-providing bacteria and deserves further exploration.

5. Conclusion

Our data confirms previous results that the Pacific oyster, Southern Australian scallops as well as Goolwa cockles – a species not previously assessed for its B_{12} content – all contain high concentrations of B_{12}. As humans cannot synthesize B_{12}, the fact that bivalves provide a high source of B_{12} is particularly important in areas of the world where access to animal proteins is expensive, or for people who want to reduce their consumption of farmed animals or fish for ethical and environmental concerns. Our research, which is one of the first studies to assess B_{12} concentrations in different tissues across species, suggests that B_{12} concentrations vary between species and among tissues within those species so B_{12} availability in shellfish foods depends upon which species and which part of the shellfish is commonly consumed (e.g., with scallops, often only the adductor muscle is consumed).

The source of such high levels of B_{12} in bivalves is still unknown, as are the metabolic processes that utilise this important vitamin. Our hypothesis that bivalves might obtain their B_{12} through their diet could not be confirmed, given that B_{12} concentrations did not increase in oysters after providing B_{12}-enriched feed. This research therefore raises further questions regarding potential B_{12} production by the animal's gill microbiome or gut; in terrestrial ruminants, the production of B_{12} by the gut microbiome is widely recognised, and it is possible that shellfish are functioning similarly in the marine environment ('marine ruminants'). Prior to being fed microalgae, D-shelled larvae already contained high amounts of B_{12}, indicating that B_{12} could have been provided by symbiotic microorganisms acquired during the early trochophore larval stage. However, B_{12} concentrations in the digestive tract of adult oysters were one of the lowest of all oyster tissues, thus contradicting this theory that B_{12} production is likely occurring in the gut microbiome. Further investigations of core microbiome communities in the gut and other tissues such as gills in relation to known B_{12}-producing bacteria, as well as experiments using B_{12} inhibitors to block B_{12} uptake or utilisation of B_{12}, might shed more light on the high levels of B_{12} in bivalves.

Our data also provides valuable insight into how and when to supply B_{12} in bivalve aquaculture production. Firstly, including higher amounts of B_{12} in growth media increases B_{12} concentrations in microalgae that are fed to bivalve larvae, although our data show that this does not necessarily lead to an increase in B_{12} concentrations within larvae nor does it lead to faster growth. Further research is needed to determine whether increased B_{12} supplementation might provide some health benefits to larvae when challenged with pathogens, or whether it improves survival during metamorphosis. Our preliminary data on B_{12} throughout oyster larval development and post metamorphosis suggests a high consumption of B_{12} during metamorphosis. Given that this life stage is highly susceptible to mortality, a deeper understanding of larval nutritional requirements during metamorphosis could aid their survival and wellbeing as juveniles. As a final note, our findings confirm that autoclaving algal growth media after vitamins have been added is not advisable or must be compensated for by hyper-supplementation, given that our data shows a heat-induced significant reduction of B_{12} in the medium and subsequently in algal cultures (at least for *C. calcitrans*). This common practice in hatcheries may result in suboptimal vitamin

levels and is not recommended.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738712.

Authors' contribution

Experimental design and conceptualization were generated by SV, AJ & XL. All laboratory studies and experiments were completed by SV & JS. Manuscript preparation and interpretation of data were conducted by SV, AJ, GHW, and all authors have read and approved the final manuscript.

CRediT authorship contribution statement

Susanne Vogeler: Conceptualization, Methodology, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition. **Gary H. Wikfors:** Validation, Writing – original draft. **Xiaoxu Li:** Conceptualization, Methodology, Resources. **Justine Sauvage:** Investigation. **Alyssa Joyce:** Conceptualization, Methodology, Resources, Validation, Writing – original draft, Supervision, Funding acquisition.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by a grant from the Swedish Foundation for International Cooperation in Research and Higher Education (STINT Joyce MG2020-8856) and The Swedish Environmental Research Council FORMAS (Joyce 942-201-1484). Additional funding was received from Helge Ax:son Johnsons Stiftelse (Vogeler F20-0291). For help with rearing of Pacific oyster larvae, we would like to thank Mark Gluis, Dr. Penny Ezzy-Miller and the hatchery team from SARDI.

References

Agarwal, S., Dey, S., Ghosh, B., Biswas, M., Dasgupta, J., 2019. Mechanistic basis of vitamin B12 and cobinamide salvaging by the *Vibrio species*. Biochim. Biophys. Acta 1867, 140–151.

Allen, L.H., 2008a. Causes of vitamin B12 and folate deficiency. Food Nutr. Bull. 29, S20–S34.

Allen, L.H., 2008b. How common is vitamin B-12 deficiency? Am. J. Clin. Nutr. 89, 693S–696S.

Allen, L.H., Miller, J.W., De Groot, L., Rosenberg, I.H., Smith, A.D., Refsum, H., Raiten, D.J., 2018. Biomarkers of nutrition for development (BOND): vitamin B-12 review. J. Nutr. 148, 1995S–2027S.

Arfken, A., Song, B., Allen Jr., S.K., Carnegie, R.B., 2021. Comparing larval microbiomes of the eastern oyster (*Crassostrea virginica*) raised in different hatcheries. Aquaculture 531, 735955.

Asmani, K., Petton, B., Le Grand, J., Mounier, J., Robert, R., Nicolas, J.-L., 2016. Establishment of microbiota in larval culture of Pacific oyster, *Crassostrea gigas*. Aquaculture 464, 434–444.

Bito, T., Teng, F., Watanabe, F., 2017. Bioactive compounds of edible purple laver *Porphyra* sp. (Nori). J. Agric. Food Chem. 65, 10685–10692.

Bito, T., Tanioka, Y., Watanabe, F., 2018. Characterization of vitamin B12 compounds from marine foods. Fish. Sci. 84, 747–755.

Calderón-Ospina, C.A., Nava-Mesa, M.O., 2020. B vitamins in the nervous system: current knowledge of the biochemical modes of action and synergies of thiamine, pyridoxine, and cobalamin. CNS Neurosci. Ther. 26, 5–13.

Carlucci, A.F., Silbernagel, S.B., 1969. Effect of vitamin concentrations on growth and development of vitamin-requiring algae. J. Phycol. 5, 64–67.

Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., Smith, A.G., 2005. Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438, 90–93.

Croft, M.T., Warren, M.J., Smith, A.G., 2006. Algae need their vitamins. Eukaryot. Cell 5, 1175–1183.

de Mendiburu, F., 2021. agricolae: Statistical Procedures for Agricultural Research. R Package Version 1.3-5.

Doxey, A.C., Kurtz, D.A., Lynch, M.D.J., Sauder, L.A., Neufeld, J.D., 2015. Aquatic metagenomes implicate *Thaumarchaeota* in global cobalamin production. ISME J. 9, 461–471.

Dubilier, N., Bergin, C., Lott, C., 2008. Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. Nat. Rev. Microbiol. 6, 725–740.

Eichel, J., González, J.C., Hotze, M., Matthews, R.G., Schröder, J., 1995. Vitamin-B12-independent methionine synthase from a higher plant (*Catharanthus Roseus*) molecular characterization, regulation, heterologous expression, and enzyme properties. Eur. J. Biochem. 230, 1053–1058.

Ellis, K.A., Cohen, N.R., Moreno, C., Marchetti, A., 2017. Cobalamin-independent methionine synthase distribution and influence on vitamin B12 growth requirements in marine diatoms. Protist 168, 32–47.

Fang, H., Kang, J., Zhang, D., 2017. Microbial production of vitamin B12: a review and future perspectives. Microbiol. Cell. Fact. 16, 15.

Froese, D.S., Fowler, B., Baumgartner, M.R., 2019. Vitamin B12, folate, and the methionine remethylation cycle—biochemistry, pathways, and regulation. J. Inherit. Metab. Dis. 42, 673–685.

Gille, D., Schmid, A., 2015. Vitamin B12 in meat and dairy products. Nutr. Rev. 73, 106–115.

Grant, M.A.A., Kazamia, E., Cicuta, P., Smith, A.G., 2014. Direct exchange of vitamin B12 is demonstrated by modelling the growth dynamics of algal–bacterial cocultures. ISME J. 8, 1418–1427.

Green, R., Allen, L.H., Bjørke-Monsen, A.-L., Brito, A., Guéant, J.-L., Miller, J.W., Yajnik, C., 2017. Vitamin B12 deficiency. Nat. Rev. Dis. Primers 3, 17040.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Guillard, R.R.L. (Ed.), Culture of Marine Invertebrate Animals. Springer, pp. 29–60.

Heal, K.R., Qin, W., Ribalet, F., Bertagnolli, A.D., Coyote-Maestas, W., Hmelo, L.R., Stahl, D.A., 2017. Two distinct pools of B12 analogs reveal community interdependencies in the ocean. Proc. Natl. Acad. Sci. U. S. A. 114, 364–369.

Helliwell, K.E., Lawrence, A.D., Holzer, A., Kudahl, U.J., Sasso, S., Kräutler, B., Smith, A. G., 2016. Cyanobacteria and eukaryotic algae use different chemical variants of vitamin B12. Curr. Biol. 26, 999–1008.

Helm, M.M., Bourne, N., Lovatelli, A., 2004. Hatchery culture of bivalves. A practical manual. FAO, Rome.

Hernández-Zárate, G., Olmos-Soto, J., 2006. Identification of bacterial diversity in the oyster *Crassostrea gigas* by fluorescent in situ hybridization and polymerase chain reaction. J. Appl. Microbiol. 100, 664–672.

Institute of Medicine, 1998. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B(6), folate, vitamin B(12), pantothenic acid, biotin, and choline. National Academies Press, Washington, DC.

Jee, P., Fernandez, L., Perkins, S.L., Brooks, S.P.J., 2014. Effect of storage and repeated freeze/thaw on (S) vitamin B12. Clin. Biochem. 47, 344.

Kazamia, E., Czesnick, H., Nguyen, T.T.V., Croft, M.T., Sherwood, E., Sasso, S., Smith, A. G., 2012. Mutualistic interactions between vitamin B12-dependent algae and heterotrophic bacteria exhibit regulation. Environ. Microbiol. 14, 1466–1476.

Keshavarz, H., Moghadam, R.S.G., 2017. Seed priming with cobalamin (vitamin B12) provides significant protection against salinity stress in the common bean. Rhizosphere 3, 143–149.

King, G.M., Judd, C., Kuske, C.R., Smith, C., 2012. Analysis of stomach and gut microbiomes of the eastern oyster (*Crassostrea virginica*) from coastal Louisiana, USA. PLoS One 7, e51475.

Kintsu, H., Nishimura, R., Negishi, L., Kuriyama, I., Tsuchihashi, Y., Zhu, L., Suzuki, M., 2020. Identification of methionine -rich insoluble proteins in the shell of the pearl oyster, *Pinctada fucata*. Sci. Rep. 10, 18335.

Koury, M.J., Ponka, P., 2004. New insights into erythropoiesis: the roles of folate, vitamin B12, and iron. Annu. Rev. Nutr. 24, 105–131.

Krichnavaruk, S., Loataweesup, W., Powtongsook, S., Pavasant, P., 2005. Optimal growth conditions and the cultivation of *Chaetoceros calcitrans* in airlift photobioreactor. Chem. Eng. J. 105, 91–98.

Laroche, O., Symonds, J.E., Smith, K.F., Banks, J.C., Mae, H., Bowman, J.P., Pochon, X., 2018. Understanding bacterial communities for informed biosecurity and improved larval survival in Pacific oysters. Aquaculture 497, 164–173.

Lees, L., Bland, A.M., DiTullio, G.R., Janech, M.G., Lee, P.A., 2020. Effect of flow rate and freezing on cyanocobalamin recovery using a commercial solid phase extraction cartridge. Ann. Mar. Sci. 4 (1), 014–018.

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N., 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Front. Microbiol. 5, 207.

Marie, B., Joubert, C., Belliard, C., Tayale, A., Zanella-Cléon, I., Marin, F., Montagnani, C., 2012. Characterization of MRNP34, a novel methionine-rich nacre protein from the pearl oysters. Amino Acids 42, 2009–2017.

Maxwell, B.E., 1952. The distribution of vitamin B12-active substances in some marine invertebrates of British Columbia. J. Fish. Res. Board Can. 9, 164–168.

Mikkelsen, K., Apostolopoulos, V., 2019. Vitamin B12, folic acid, and the immune system. In: Mahmoudi, M., Rezaei, N. (Eds.), Nutrition and Immunity. Springer, pp. 103–114.

Miyamoto, E., Tanioka, Y., Nakao, T., Barla, F., Inui, H., Fujita, T., Nakano, Y., 2006. Purification and characterization of a corrinoid-compound in an edible cyanobacterium *Aphanizomenon flos-aquae* as a nutritional supplementary food. J. Agric. Food Chem. 54, 9604–9607.

Moll, R., Davis, B., 2017. Iron, vitamin B12 and folate. Medicine 45, 198–203.

Na, H., Ponomarova, O., Giese, G.E., Walhout, A.J.M., 2018. *C. elegans* MRP-5 exports vitamin B12 from mother to offspring to support embryonic development. Cell Rep. 22, 3126–3133.
Nef, C., Jung, S., Mairet, F., Kaas, R., Grizeau, D., Garnier, M., 2019. How haptophytes microalgae mitigate vitamin B12 limitation. Sci. Rep. 9, 8417.
O'Connor, R.M., Fung, J.M., Sharp, K.H., Benner, J.S., McClung, C., Cushing, S., Distel, D. L., 2014. Gill bacteria enable a novel digestive strategy in a wood-feeding mollusk. Proc. Natl. Acad. Sci. U. S. A. 111, E5096–E5104.
Okbamichael, M., Sañudo-Wilhelmy, S.A., 2004. A new method for the determination of vitamin B12 in seawater. Anal. Chim. Acta 517, 33–38.
Ortigues-Marty, I., Micol, D., Prache, S., Dozias, D., Girard, C.L., 2005. Nutritional value of meat: the influence of nutrition and physical activity on vitamin B12 concentrations in ruminant tissues. Reprod. Nutr. Dev. 45, 453–467.
Panzeca, C., Beck, A.J., Tovar-Sanchez, A., Segovia-Zavala, J., Taylor, G.T., Gobler, C.J., Sañudo-Wilhelmy, S.A., 2009. Distributions of dissolved vitamin B12 and Co in coastal and open-ocean environments. Estuar. Coast. Shelf Sci. 85, 223–230.
Paul, C., Brady, D.M., 2017. Comparative bioavailability and utilization of particular forms of B12 supplements with potential to mitigate B12-related genetic polymorphisms. Integr. Med. 16, 42–49.
Pierce, M.L., Ward, J.E., 2018. Microbial ecology of the bivalvia, with an emphasis on the family ostreidae. J. Shellfish Res. 37, 793–806.
Provasoli, L., Carlucci, A., 1974. Vitamins and growth regulators. Bot. Monogr. 10.
Provasoli, L., McLaughlin, J.J.A., Droop, M.R., 1957. The development of artificial media for marine algae. Arch. Mikrobiol. 25, 392–428.
R Core Team, 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.
Rodionov, D.A., Vitreschak, A.G., Mironov, A.A., Gelfand, M.S., 2003. Comparative genomics of the vitamin B12 metabolism and regulation in prokaryotes. J. Biol. Chem. 278, 41148–41159.
Roeselers, G., Newton, I.L.G., 2012. On the evolutionary ecology of symbioses between chemosynthetic bacteria and bivalves. Appl. Microbiol. Biotechnol. 94, 1–10.
Roth, J.R., Lawrence, J.G., Bobik, T.A., 1996. Cobalamin (coenzyme B12): synthesis and biological significance. Annu. Rev. Microbiol. 50, 137–181.
Sahari, M.A., Ahmadnia, A., Barzegar, M., Noorolahi, Z., 2014. Vitamin losses during frozen storage of *Liza aurata* (Risso, 1810), *Cyprinus carpio* L. 1758, *Clupeonella cultriventris caspia* (Nordmann, 1840), *Rutilus frisii kutum* (Kamenskii, 1901) and *Sander lucioperca* (L., 1758). J. Appl. Ichthyol. 30, 366–371.
Sañudo-Wilhelmy, S.A., Gómez-Consarnau, L., Suffridge, C., Webb, E.A., 2014. The role of B vitamins in marine biogeochemistry. Ann. Rev. Mar. Sci. 6, 339–367.
Seguineau, C., Migaud, H., Quere, C., Moal, J., Samain, J.-F., 2001a. Changes in tissue concentrations of the vitamins B1 and B2 during reproductive cycle of bivalves: part 2. The Pacific oyster *Crassostrea gigas*. Aquaculture 196, 139–150.
Seguineau, C., Saout, C., Paulet, Y.-M., Muzellec, M.-L., Quéré, C., Moal, J., Samain, J.-F., 2001b. Changes in tissue concentrations of the vitamins B1 and B2 during reproductive cycle of bivalves: part 1: the scallop *Pecten maximus*. Aquaculture 196, 125–137.
Shelton, A.N., Seth, E.C., Mok, K.C., Han, A.W., Jackson, S.N., Haft, D.R., Taga, M.E., 2018. Uneven distribution of cobamide biosynthesis and dependence in bacteria predicted by comparative genomics. ISME J. 13, 789–804.
Shiau, S.-Y., Lung, C.-Q., 1993. Estimation of the vitamin B12 requirement of the grass shrimp, *Penaeus monodon*. Aquaculture 117, 157–163.
Stabler, S.P., Allen, R.H., 2004. Vitamin B12 deficiency as a worldwide problem. Annu. Rev. Nutr. 24, 299–326.
Stevick, R.J., Sohn, S., Modak, T.H., Nelson, D.R., Rowley, D.C., Tammi, K., Gómez-Chiarri, M., 2019. Bacterial community dynamics in an oyster hatchery in response to probiotic treatment. Front. Microbiol. 10.
Stravoravdis, S., Shipway, J.R., Goodell, B., 2021. How do shipworms eat wood? Screening shipworm gill symbiont genomes for lignin-modifying enzymes. Front. Microbiol. 12, 665001.
Sugita, H., Iwata, M., Kato, S., Sugiura, C., Ueda, R., Deguchi, Y., 1991. Vitamin B12-producing ability of the gut microflora of marine gastropods. Aquacult. Sci. 39, 363–369.
Suzuki, M., Kubota, K., Nishimura, R., Negishi, L., Komatsu, K., Kagi, H., Weiner, S., 2019. A unique methionine-rich protein–aragonite crystal complex: structure and mechanical functions of the *Pinctada fucata* bivalve hinge ligament. Acta Biomater. 100, 1–9.
Tandon, P., Jin, Q., Huang, L., 2017. A promising approach to enhance microalgae productivity by exogenous supply of vitamins. Microbiol. Cell. Fact. 16, 219.
Tanioka, Y., Takenaka, S., Furusho, T., Yabuta, Y., Nakano, Y., Watanabe, F., 2014. Identification of vitamin B12 and pseudovitamin B12 from various edible shellfish using liquid chromatography–electrospray ionization/tandem mass spectrometry. Fish. Sci. 80, 1065–1071.
Teng, F., Tanioka, Y., Hamaguchi, N., Bito, T., Takenaka, S., Yabuta, Y., Watanabe, F., 2015. Determination and characterization of vitamin B12 compounds in edible sea snails, ivory shell Babylonia japonica and turban shell *Turdo Batillus cornutus*. Fish. Sci. 81, 1105–1111.
Trabal Fernández, N., Mazón-Suástegui, J.M., Vázquez-Juárez, R., Ascencio-Valle, F., Romero, J., 2014. Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) during commercial production. FEMS Microbiol. Ecol. 88, 69–83.
Ueta, K., Nishioka, M., Yabuta, Y., Watanabe, F., 2010. TLC-bioautography analysis of vitamin B12 compound from the short-necked clam (*Ruditapes philippinarum*) extract used as a flavoring. J. Liq. Chromatogr. Relat. Technol. 33, 972–979.
Ueta, K., Ishihara, Y., Yabuta, Y., Masuda, S., Watanabe, F., 2011. TLC-analysis of a corrinoid compound from Japanese rock-oyster "Iwa-gaki"(*Crassostrea nippona*). J. Liq. Chromatogr. Relat. Technol. 34, 928–935.
Van De Lagemaat, E.E., De Groot, L.C., Van Den Heuvel, E.G., 2019. Vitamin B12 in relation to oxidative stress: a systematic review. Nutrients 11, 482.
Vignier, J., Laroche, O., Rolton, A., Wadsworth, P., Kumanan, K., Trochel, B., King, N., 2021. Dietary exposure of Pacific oyster (*Crassostrea gigas*) larvae to compromised microalgae results in impaired fitness and microbiome shift. Front. Microbiol. 12, 706214.
Vitreschak, A.G., Rodionov, D.A., Mironov, A.A., Gelfand, M.S., 2003. Regulation of the vitamin B12 metabolism and transport in bacteria by a conserved RNA structural element. RNA 9, 1084–1097.
Vogeler, S., Miller-Ezzy, P., Li, X., Wikfors, G.H., Joyce, A., 2018. First report of a putative involvement of the NMDA pathway in Pacific oyster (*Crassostrea gigas*) development: effect of NMDA receptor ligands on oyster metamorphosis with implications for bivalve hatchery management. Aquaculture 497, 140–146.
Wagner-Döbler, I., Ballhausen, B., Berger, M., Brinkhoff, T., Buchholz, I., Bunk, B., Gerdts, G., 2010. The complete genome sequence of the algal symbiont *Dinoroseobacter shibae*: a hitchhiker's guide to life in the sea. ISME J. 4, 61–77.
Wallace, R.K., Waters, P., Rikard, F.S., 2008. Oyster hatchery techniques. Southern Regional Aquaculture Center, Stoneville, MS.
Walne, P.R., 1970. Studies on the food value of nineteen genera of algae to juvenile bivalves of the genera *Ostrea*, *Crassostrea*, *Mercenaria* and *Mytilus*. Fish. Invest. Ser. 2 (26), 1–62.
Watanabe, F., Katsura, H., Takenaka, S., Fujita, T., Abe, K., Tamura, Y., Nakano, Y., 1999. Pseudovitamin B12 is the predominant cobamide of an algal health food, spirulina tablets. J. Agric. Food Chem. 47, 4736–4741.
Watanabe, F., Katsura, H., Takenaka, S., Enomoto, T., Miyamoto, E., Nakatsuka, T., Nakano, Y., 2001. Characterization of vitamin B12 compounds from edible shellfish, clam, oyster, and mussel. Int. J. Food Sci. Nutr. 52, 263–268.
Wegner, K.M., Volkenborn, N., Peter, H., Eiler, A., 2013. Disturbance induced decoupling between host genetics and composition of the associated microbiome. BMC Microbiol. 13, 1–12.
Wei, J., Yu, N., Tian, W., Zhang, F., Wu, Q., Li, E., Chen, L., 2014. Dietary vitamin B12 requirement and its effect on non-specific immunity and disease resistance in juvenile Chinese mitten crab *Eriocheir sinensis*. Aquaculture 434, 179–183.
Wilson, H.R., 1997. Effects of maternal nutrition on hatchability. Poult. Sci. 76, 134–143.
Yuasa, M., Kawabeta, K., Eguchi, A., Abe, H., Yamashita, E., Koba, K., Tominaga, M., 2018. Characterization of taste and micronutrient content of rock oysters (*Crassostrea nippona*) and Pacific oysters (*Crassostrea gigas*) in Japan. Int. J. Gastro. Food Sci. 13, 52–57.