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Lipid and Astaxanthin Contents and Biochemical Post-Harvest Stability in *Calanus finmarchicus*

Thesis for the degree of Philosophiae Doctor

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Figure 1: A happy crew with the catch of the day (Photo: Jan Ove Evjemo)

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Summary

Calanus finmarchicus is a new marine species of commercial interest because of its high abundance in Northern areas and its biochemical components. The aquaculture feed industry needs new marine ingredients, most of all n-3 polyunsaturated fatty acids (PUFA). As the proportion of marine fish stocks that are either fully exploited, over exploited or depleted is 85% (FAO, 2010), further growth of the aquaculture industry requires new sources of n-3 PUFA. The aim of the present study was to explore the contents and variability of lipids and astaxanthin in *C. finmarchicus*, both in live populations in natural waters and post-harvest. We need to better understand these variabilities to obtain biomass with high value and quality. The content of lipids and astaxanthin was studied in the early spring in the Trondheimsfjord and off the coast of Norway. The effects of heat stabilisation, storage and freeze drying on the biochemical content in harvested *C. finmarchicus* were studied.

The content of lipids in *C. finmarchicus* collected from January to June was studied over a three-year period (2009-2011). The content and composition of n-3 PUFA in *C. finmarchicus* reflected the content of n-3 PUFA in the phytoplankton, and the dominant fatty acids were C18:4 n-3, C20:5 n-3 and C22:6 n-3. The content of the monounsaturated fatty acids (MUFA) C20:1 n-9 and C22:1 n-11 in *C. finmarchicus* was low in surface waters. The content of C14:0 and C16:0 did not increase in *C. finmarchicus* in the spring, suggesting the use of dietary fatty acids as precursors for the synthesis of fatty alcohols. The total fatty acid content in the specimens increased from February to June; they were smaller and leaner in surface waters (0-50 m) compared to those in deeper waters (300-440 m). These results suggests that the onset of vertical migration of stage V of *C. finmarchicus* was triggered by a high lipid level. Stage V that do not reach this lipid level during the phytoplankton bloom is suggested to moult to females and start a new generation.

The content of astaxanthin in *C. finmarchicus* off the coast of Norway increased from March to June in 2008, but showed high daily variations. The fraction of astaxanthin esters of total astaxanthin also increased during the same time period. During a short-term study in the early spring off the coast of central Norway in 2009, the total content of astaxanthin in *C. finmarchicus* showed little variation. However, the fraction of free astaxanthin was reduced by 50% during the 30 h study. No correlations of total astaxanthin were found with depth, but a negative correlation was found for total astaxanthin and the fraction of females in the samples, indicating a lower content of astaxanthin in females compared to in stages IV and V of *C. finmarchicus*. Astaxanthin is thought to have a photo-protective role in copepods, and the increasing content of astaxanthin found in the spring could suggest an increased need for photo-protection with increasing solar irradiation during the lipid accumulation period. The day to day variations are not ex-

plained by changes in the light conditions, but are rather a result of feeding and further synthesis of astaxanthin.

The autolytic enzymes of *C. finmarchicus* will lead to *post mortem* alterations in the lipids and proteins of *C. finmarchicus*. Heating at 70°C for 5 min inactivated the lipolytic and proteolytic enzymes in *C. finmarchicus*. The temperature was rapidly increased from 0°C to 70°C. It is important to avoid degradation by enzymes during the process of heating, as the optimum temperature for the activity of several enzymes is around 50°C. Storage of untreated biomass at -20°C resulted in the loss of all phospholipids and the formation of free fatty acids within 4 months. In heat-treated biomass, the content of phospholipids was maintained and the content of free fatty acids remained low and stable during 12 months of storage.

Vacuum freeze drying is a common way to preserve food. In this study, different freeze drying processes (vacuum, atmospheric and nitrogen) were evaluated as preservation methods for *C. finmarchicus*. Freeze drying of the biomass resulted in a reduced phospholipid content. This could have been caused by the pre-treatment, which included grinding the biomass at -10°C. Drying under nitrogen led to a complete loss of phospholipids, the formation of free fatty acids and a high content of secondary oxidation products and was probably caused by a prolonged drying time compared to the other drying methods. Atmospheric freeze drying gave a dry product within 24 h and showed the lowest loss of phospholipids and further resulted in the best quality of biomass from *C. finmarchicus* of all the drying procedures tested.

The activity of autolytic enzymes is challenging to control by freezing and freeze drying; time and temperature are probably the most important factors when trying to control autolytic activity in *C. finmarchicus*. However, to obtain a stable biomass with a maintained content of phospholipids, heating would be a good preservation method for *C. finmarchicus*. The biomass should be heat treated directly after harvest to maintain the content of phospholipids. Rapid freezing of the biomass will prevent degradation if heating is difficult to obtain on the vessel, but freezing over longer time is not a good alternative for preservation.

Contents

List of papers

Paper I

Bergvik, M., Leiknes, Ø., Altin, D., Dahl, K. R. and Olsen, Y., Dynamics in lipid contents and biomass of *Calanus finmarchicus* in a Norwegian fjord. Submitted.

Paper II

Bergvik, M., Overrein, I., Altin, D., Østerlie, M., Evjemo, J. O. and Olsen, Y., Astaxanthin content in *Calanus finmarchicus* off the coast of Norway. Submitted.

Paper III

Bergvik, M., Overrein, I., Bantle, M., Evjemo, J. O. and Rustad, T. (2012), Properties of *Calanus finmarchicus* biomass during frozen storage after heat inactivation of autolytic enzymes, Food Chemistry, 132: 209-215.

Paper IV

Bantle, M., Bergvik, M. and Rustad, T. (2010), Lipid class changes and lipid oxidation in *Calanus finmarchicus* during vacuum, atmospheric and nitrogen freeze drying. Proceedings 17th International Drying Symposium, 2010: Magdeburg, Germany: p. 1907-1914.

Contributions

I performed all work on which this thesis is based. Øystein Leiknes contributed to the planning, was a major part of collecting the samples and was involved in the final stages of writing Paper I. Kjersti Rennan Dahl developed a method of lipid analysis and did the major part of the lipid analyses in Paper I. Dag Altin contributed to the planning and was involved in the final stages of writing Paper I and II. He also was a part of the analysis in Paper I. Ingrid Overrein contributed to planning, collecting samples and was involved in the final stages of writing Paper II and III. She also contributed to analyses in Paper III. Marianne Østerlie contributed to the analyses and was involved in the final stages of writing Paper II. Jan Ove Evjemo contributed to collection of samples and was involved in the final stages of writing Paper II and III. Michael Bantle planned Paper IV and was a part of planning Paper III. He was a part of collecting the samples of Paper III and IV, was a part of writing paper IV and was involved in the final stages of writing Paper III. Turid Rustad was a part of the analysis of Paper III and was involved in the final stages of writing Paper III and IV. Yngvar Olsen contributed to planning and was involved in the final stages of writing Paper I and Paper II.

Abbreviations

CI-CV	Copepodite stage I - copepodite stage V
CHO	Cholesterol
DAG	Diacylglyceride
FFA	Free fatty acids
FID	Flame ionisation detector
GC	Gas chromatography
HPLC	High performance liquid chromatography
LC-PUFA	Long chain polyunsaturated fatty acids ($\geq$20 carbon)
MAG	Monoacylglyceride
PC	Phosphatidyl choline
PE	Phosphatidyl ethanolamine
PL	Phospholipids
PUFA	Polyunsaturated fatty acids ($\geq$18 carbon)
RF	Response factor
TAG	Triacylglyceride
TLC	Thin layer chromatography
WE	Wax esters

Chapter 1

Introduction

The two largest zooplankton stocks on earth are the Antarctic krill (*Euphausia superba*) found in the Southern Ocean (Siegel et al., 1998) and *Calanus finmarchicus* in the Northern Atlantic Ocean (Planque and Batten, 2000). While there is a substantial harvest of *E. superba* in Antarctic waters, *C. finmarchicus* has not been exploited to any extent other than for research. *C. finmarchicus* is a new marine species of commercial interest due to its biochemical components and its high production; the estimated production in the Nordic Sea is estimated at 74 million tons yr^{-1} (Aksnes and Blindheim, 1996). *C. finmarchicus* builds up large lipid reserves in the spring and may contain high fractions of eicosapentaneoic acid (C20:5 n-3) and docosahexaenoic acid (C22:6 n-3) in addition to the n-3 polyunsaturated fatty acid (PUFA) stearidonic acid (C18:4 n-3). Wax esters (WE) serve as the storage lipid in *C. finmarchicus*, which is a common lipid class in herbivorous zooplankton that experience short periods with high food abundance followed by long periods of food shortage (Sargent et al., 1981).

Global aquaculture has grown steadily in the last 50 years, and the FAO (2010) has reported that the per capita supply of food fish from aquaculture has shown an annual growth rate of 6.6 percent since 1970. The FAO (2010) further reported that 85% of marine fish stocks are either fully exploited, overexploited or depleted. Further growth of the aquaculture sector requires alternative feed sources. Vegetable sources can replace part of the fish meal and oil, but the need for n-3 long chain PUFA (LC-PUFA) like C22:6 n-3 and C20:5 n-3 will require new marine lipid sources (Turchini et al., 2009; Olsen, 2011). Harvesting of zooplankton could be one alternative to meet the increased demand for n-3 PUFA in the aquaculture industry.

1.1 Ingredients and applications of *C. finmarchicus*

Wax esters (WE) are the main component of *C. finmarchicus* and can comprise up to 50% of the dry weight. Marine waxes are composed of long chain polyunsaturated fatty acids (LC-PUFA) and long chain mono-unsaturated fatty alcohols (MUFA), which have distinct properties. The demand for these components was one of the reasons for the increase in the whaling industry during the 1950s. The oil of the sperm whale contains 70% WE and was used as a lubricant, in cosmetics and as a chemical raw material for the production of soaps and detergents (Sargent et al., 1976). The demand for WE nearly resulted in the extinction of the sperm whale, and led to the end of whaling this species.

Some fish species have to some extent been harvested to provide waxes, while Sargent et al. (1976) claimed that copepods are the greatest global reserves of wax esters. Wax esters from copepods are also interesting as a feed ingredient in the aquaculture industry. Atlantic salmon (*Salmo salar*) has been shown to have some challenges with digesting wax esters compared to triacylglycerols (TAG), although *Calanus* is the natural pray of Atlantic salmon (Bogevik, 2011). Bogevik (2011) further concluded that with medium inclusion rates of *Calanus* oil in the diets the digestion is similar as compared to fish oil inclusion. Several studies show that Atlantic salmon are able to utilise *Calanus* oil in their diets (Olsen et al., 2004; Bogevik et al., 2009; Hynes et al., 2009; Oxley et al., 2009), demonstrating the suitability of wax esters from *C. finmarchicus* as a new marine ingredient in fish feed. The digestion of wax esters in fish needs to be further studied.

Humans also experience some challenges regarding the digestion of wax esters. Recent studies with *Calanus* oil supplements have shown positive health effects in mice (Eilertsen et al., 2012), and an ongoing study with wax esters from *C. finmarchicus* as a food supplement for humans could provide more information on their effects on human health (http://clinicaltrials.gov, University of Tromsø).

Phospholipids (PL) are not a major fraction of the lipids of *C. finmarchicus,* but they still represent unique lipids. The main PL are phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) (Farkas et al., 1988; Overrein, 2010; Paper III; Paper IV). The total content of C20:5 n-3 and C22:6 n-3 is high in PL (Farkas et al., 1988; Albers et al., 1996; Scott et al., 2002) and could meet the dietary demands of early marine fish larvae, as they require phospholipids (Tocher et al., 2008) in addition to a high content of C22:6 n-3 and C20:5 n-3 (Sargent et al., 2002). There has been an increasing awareness of the importance of marine lipids in human nutrition as well, and the beneficial health effects of consumption of n-3 LC-PUFA, which are mainly provided by marine lipids, have been documented. C22:6 n-3 and C20:5 n-3 are LC-PUFA that play vital roles in membrane fluidity, cellular signalling and gene expression. They have been shown to provide positive benefits in coronary heart disease, diabetes, immune disorders and mental health (Larsen et al., 2011).

C. finmarchicus contains the carotenoid astaxanthin (Foss et al., 1987). Astaxanthin is an important and expensive feed ingredient in feed for the cultivation of salmonoids and crustaceans (Higuera-Ciapara et al., 2006). The colouration of these farmed species is an important quality criterion for consumers. The red colour in crustaceans and salmonoids is a result of the caroteniod content in the feed, and this pigment must be added to the diets of these species. Today, synthetic astaxanthin dominates the world market; however, there is an increasing interest in natural sources of this pigment (Linan-Cabello et al., 2002; Higuera-Ciapara et al., 2006). The astaxanthin in *C. finmarchicus* is highly esterified (70-80%) with fatty acids. Hynes et al. (2009) studied *Calanus* oil as a natural source for flesh pigmentation in Atlantic salmon (*Salmo salar* L.) compared to a synthetic source of astaxanthin. They reported that the content of natural pigment had to be higher in the feed, compared to the synthetic astaxanthin, to obtain a colouration in the flesh that is preferred to the market. However, the authors conclude with promising results in the use of *Calanus* oil in feeds of Atlantic salmon. More studies are needed to reveal the potential of *C. finmarchicus* as a source of astaxanthin in feeds for Atlantic salmon.

Seafood has been shown to contain protein with all the essential amino acids, have high digestibility and the smaller peptides have been shown to possess bio-active properties (Larsen et al., 2011). The amino acid composition of the crude protein in *C. finmarchicus*

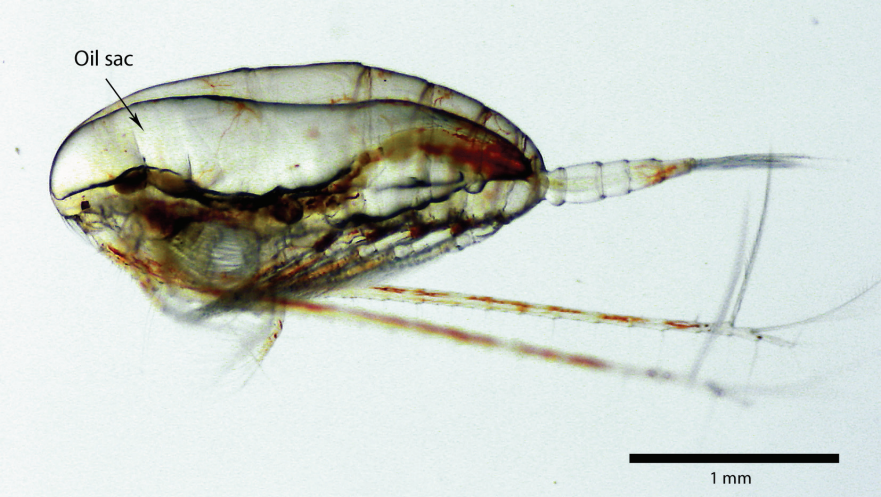

Figure 1.1: *Calanus finmarchicus* copepodite stage V (Photo: Dag Altin)

has also been shown to contain all the essential amino acids (Cowey, 1963; Overrein, 2010). Enzymatic activity in *C. finmarchicus* can lead to the degradation of proteins to smaller peptides and free amino acids. In aquaculture, an increased content of peptides and free amino acids in the feed can provide a positive effect on growth or survival for some marine species (Kolkovski, 2001).

C. finmarchicus has a range of autolytic enzymes that cause the deterioration of biomass *post mortem*. Solgaard et al. (2007) identified three classes of proteases, which were serine, metallo and aspartic proteases. *C. finmarchicus* also contains lipases and phospholipases, but only general lipase and phospholipase activity has been studied (Paper III). Marine enzymes can be utilised commercially as biotechnological tools in the production of fishery products (Gildberg et al., 2000).

A major part of the exoskeleton in crustaceans is chitin, which was found to constitute 5% of the dry weight in *C. finmarchicus* (Båmstedt, 1986). Chitin can be chemically transformed to chitosan, which is a major commercial chemical used in medicine and cosmetics.

The biomass of *C. finmarchicus* itself is also interesting as a marine aroma and flavour ingredient.

1.2 Life of *C. finmarchicus*

C. finmarchicus has a complex life cycle with six naupliar stages and five copepodite stages before it becomes an adult. The females spawn at the time of the spring bloom, and the offspring feed mainly on phytoplankton (Tande and Hopkins, 1981; Marshall and Orr, 1955); *C. finmarchicus* is, accordingly, mainly herbivorous (Falk-Petersen et al., 1987; Saage et al., 2008). Specimens accumulate lipids for storage mainly in the copepodite stages IV and V (CIV and CV). The storage lipid is located in an oil sac situated along the prosome (Fig. 1.1) and contains WE (Miller et al., 1998). They also accumulate a

small fraction of triacylglycerides (TAG), which have been shown to function as a short-term lipid storage (Sargent and Henderson, 1986; Jonasdottir, 1999). *C. finmarchicus* starts to descend to deeper waters for dormancy in the summer and stay dormant until the late winter, mainly as CV. The copepods then ascend to surface waters in the late winter and moult into adults (Marshall and Orr, 1955). *C. finmarchicus* has been shown to have a generation time of 1-2 years off the Norwegian coast (Tande and Hopkins, 1981; Diel and Tande, 1992).

1.3 Dynamics of lipids and astaxanthin

C. finmarchicus feeds mainly on phytoplankton, and the production of copepods is therefore naturally reflected in the quantity and quality of phytoplankton production. Phytoplankton can contain high fractions of n-3 PUFA and carotenoids, and the contents of these components in *C. finmarchicus* can therefore be high. The content of n-3 PUFA and astaxanthin are the main characteristics that make this zooplankton interesting for commercial exploitation. It is therefore important to study the changes in these components in natural populations of *C. finmarchicus* in the sea.

1.3.1 Lipid and fatty acid content

The fatty acids found in the WE of *C. finmarchicus* is partly reflected by the fatty acid composition of the dietary phytoplankton (Lee et al., 1971a; Kattner, 1989; Graeve et al., 1994). However, advection results in copepods with different origins, which leads to spatial and depth variations in the fatty acid content (Kattner et al., 1989).

The variability of fatty acids in WE is high; Table 1.1 show examples of the variation in n-3 PUFA. The contents of C18:4 n-3, C20:5 n-3 and C22:6 n-3 can occasionally be close to zero in the WE of *C. finmarchicus* (Kattner et al., 1989; Kattner and Krause, 1989). WE can also contain high fractions of these fatty acids, for example C18:4 n-3, C20:5 n-3 and C22:6 n-3 can comprise 26%, 19% and 5%, respectively, of total fatty acids (TFA) (Table 1.1).

Typical fatty acids of dinoflagellates and flagellates such as *Phaeocystis pouchetii* are C18:4 n-3 and C22:6 n-3 (Harrington et al., 1970; Reitan et al., 1994; Hamm et al., 2001), whilst C20:5 n-3 is a typical fatty acid in diatoms (Kates and Volcani, 1966). The content of the mono-unsaturated fatty acids (MUFA) C20:1 n-9 and C22:1 n-11, synthesised by the copepods, can constitute 4-19% and 6-24% of the TFA, respectively (Table 1.1).

WE consist of equimolar amounts of fatty acids and fatty alcohols and are synthesised by copepods. The fatty alcohols are synthesised *de novo* by copepods; the bio-synthetic route is suggested to be a reduction of the fatty acid to the corresponding fatty alcohol. The main fatty alcohols in the WE of *C. finmarchicus* are C20:1 n-9, C22:1 n-11 and C16:0, which comprise on average 90% of the total fatty alcohols (e.g. Kattner and Krause, 1989; Petursdottir et al., 2010). The fatty acids in WE will partly reflect typical phytoplankton fatty acids and can contain high amounts of C18:4 n-3, C20:3 n-3 and C22:6 n-3, but the monounsaturated fatty acids (MUFA) C20:1 n-9 and C22:1 n-11 that are synthesised *de novo* (Sargent, 1978) are also prominent components of the WE (Sargent and Henderson, 1986; Kattner and Krause, 1989). The *de novo* synthesis of fatty acids in copepods is unique. In contrast to the conventional route of lipid metabolism, where *de novo* synthesis

Table 1.1: Content of n-3 PUFA (C18:4 n-3, C20:5 n-3 and C22:6 n-3), C20:1 n-9 and C22:1 n-11 in the WE of *C. finmarchicus* sampled from natural waters. Numbers are presented as % of total fatty acids (weight).

Stage	Location	Time of year	C18:4 n-3	C20:5 n-3	C22:6 n-3	C20:1 n-9	C22:1 n-11	Reference
n.r.	Coast off Norway	Summer	24	7	2	4	5	Volkman et al. (1980)
n.r.	n.r.	Summer	23	6	0	9	13	Sargent and Henderson (1986)
n.r.	Northern Norway	All year	1-23	4-11	0.5-5	9-14	11-19	Falk-Petersen et al. (1987)
F	Arctic	n.r.	2-14	0-8	1-2	13-17	12-24	Graeve and Kattner (1992)
CV	Arctic	June	26	9	2	7	9	Graeve et al. (1994)
F	Arctic	June-July	14	11	2	8	7	Albers et al. (1996)
CV-F	Arctic	Aug-Sept	3	11	1	15	10	Scott et al. (2002)
CV-F	Arctic	All year	0-8	1-16	0-3	15-19	9-16	Falk-Petersen et al. (2009)
CIII-CVI	Coast off Norway	Spring-autumn	5-18	8-10	2	5-13	10-18	Overrein (2010)
CV	Mid-Atlantic Ridge	June	7-16	6-19	3-5	4-5	6-12	Petursdottir et al. (2010)

Abbreviations: Not reported (n.r.), Copepodite stage III - V (CIII-CV), Female (F)

Table 1.2: Content of C20:5 n-3 and C22:6 n-3 (% of TFA) in the total phospholipids of *C. finmarchicus.*

Stage	Location	Time of year	C20:5 n-3	C22:6 n-3	Reference
n.r.	Coast off Norway	Spring-Fall	24-30	40-43	Farkas et al. (1988)
F	Arctic	June-July	19	37	Albers et al. (1996)
F	Arctic	Aug-Sept	24	41	Scott et al. (2002)
CV	Arctic	Spring-winter	13	10-24	Falk-Petersen et al. (2009)
F	Arctic	Spring	23	21	Falk-Petersen et al. (2009)
CIII-CVI	Coast off Norway	Spring-autumn	21	42	Overrein (2010)

Abbreviations: Not reported (n.r.), Female (F), Copepodite stage V (CV)

of fatty acids is inhibited by the accumulation of dietary fatty acids, *C. finmarchicus* has the ability to synthesise fatty acids simultaneously while accumulating dietary fatty acids (Sargent and Henderson, 1986).

Phospholipids are tightly regulated in *C. finmarchicus,* because the content of fatty acids has been shown to be quite stable, compared to that of WE. Phospholipids contain large fractions of C20:5 n-3 and C22:6 n-3, which comprise 50-70% of total fatty acids in PL (Table 1.2). Several studies have shown an average content of 20% of C20:5 n-3 and 40% of C22:6 n-3, while the ratio between C20:5 n-3 and C22:6 n-3 was around 1:2 (Farkas et al., 1988; Fraser et al., 1989; Overrein, 2010). This relationship has also been shown to be 1:2 in *C. helgolandicus* (Lee et al., 1971b). However, Falk-Petersen et al. (2009) reported some earlier unpublished data of PL fatty acids in CV and in females. They found lower fractions of C20:5 n-3 and C22:6 n-3 for copepodite stage V compared to females, in addition to a ratio of 1:1 between C20:5 n2 and C22:6 n-3. The dominating phospholipids in *C. finmarchicus* are mainly phosphatidyl choline (PC) and phosphatidyl etanolamine (PE) (Farkas et al., 1988; Overrein, 2010; Paper III; Paper IV). The content of C20:5 n-3 and C22:6 n-3 in PC were found to be 31% and 36% respectively, and in PE they comprised 6% and 56% C20:5 n-3 and C22:6 n-3, respectively (Overrein, 2010).

1.3.2 Astaxanthin content

Copepods cannot synthesise carotenoids *de novo*, and are dependent on precursors from the diet. The most common carotenoid pigment in copepods is astaxanthin (Fig. 1.2), which is a xanthophyll and a derivative of β-carotene (Goodwin, 1971). Astaxanthin is the only carotenoid that is found in *C. finmarchicus* (Foss et al., 1987), and it can be esterified with one or two fatty acids, to yield mono- and di-esters, respectively. Most of the astaxanthin in *C. finmarchicus* is present in the esterified form (Foss et al., 1987). The content of astaxanthin and its esters has been found to be highly dynamic in calanoid copepods (Juhl et al., 1996; Andersson et al., 2003; Sommer et al., 2006; Paper II). Some studies have shown a higher content of carotenoids in copepods during the night or at sunrise compared during the day, which can be explained by higher feeding activity at night (Kleppel et al., 1985; Andersson et al., 2003; Sommer et al., 2006).

Carotenoid pigmentation in zooplankton is believed to protect the animals against harmful ultraviolet (UV) radiation (Hairston, 1975). However, strong pigmentation makes the copepods more visible to predators, and Hansson (2000) has suggested that the degree of pigmentation is a trade-off between the threat of predators and UV radiation. However,

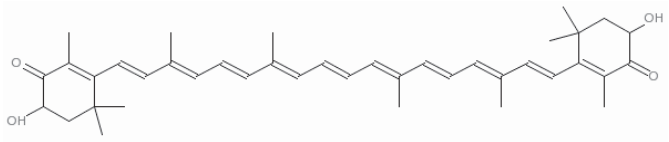

Figure 1.2: Astaxanthin

studies from natural marine waters are scarce, which makes it difficult to conclude on both the content and role of astaxanthin in copepods.

1.4 *Post mortem* changes in *C. finmarchicus*

C. finmarchicus contains a range of digestive enzymes for the catabolism of lipids, proteins and carbohydrates (Bond, 1934), and a rapid change in the lipid and protein composition is found in *C. finmarchicus post mortem* (Overrein, 2010). Phospholipids are rapidly hydrolysed, resulting in lyso-phospholipids and free fatty acids. Lyso-phospholipids are further hydrolysed to free fatty acids and glycerophosphoryl backbones. Proteolytic activity will degrade proteins to peptides and free amino acids. Solgaard et al. (2007) and Solgaard et al. (2009) found high proteolytic activity in *C. finmarchicus* with a high degree of *post mortem* degradation and subsequent leaching of nutrients as a challenge for processing the raw material. Similar problems and characteristics have been found for other zooplankton species (Grabner et al., 1981; Ohman, 1996; Sasaki and Capuzzo, 1984) and are also well-documented for krill (Ellingsen, 1982; Saether et al., 1986, 1987). Tande and Slagstad (1982) found a seasonal variation in amylase and trypsin activity, with the highest activity displayed during the spawning period in adult *C. finmarchicus.*

The high *post mortem* enzymatic activity in *C. finmarchicus* represents a major challenge during harvesting, and to avoid a reduction in quality, enzyme activity needs to be low or preferably inactivated. Freezing is a common way to preserve food and is probably the most convenient method for the preservation of biomass on a fishing vessel due to limited access to equipment (Paper III). Drying is another common way of preserving biomass that could increase the storage time (Paper IV). However, these methods do not permanently inactivate the enzymes, and their activity will increase again when the frozen biomass is thawed or when the dried biomass is rehydrated. Heat is the most common way to fully inactivate enzymes, but it requires heating equipment onboard vessels.

1.4.1 Temporary inactivation of autolytic enzymes

Freezing has been the most common way to preserve food ever since the freezer was introduced. Autolytic enzymes are less active at low temperatures and can even be denatured (Scopes, 1994). However, many enzymes are still active at temperatures below 0°C and will cause the slow degradation of lipids and proteins. The deterioration of quality in fish muscle as a result of lipolytic activity has long been recognised as a problem. Lovern and Olley (1962) found the highest content of FFA in cod flesh when stored at -4°C compared to lower and higher temperatures. Frozen storage has also been recognised as a problem for copepods and krill, because it will result in a lower content of phospholipids as a result of enzymatic activity (Sasaki and Capuzzo, 1984; Ohman, 1996; Kolakowski

and Sikorski, 2000; Paper III). However, freezing is the most readily available tool for preserving biological samples and biomass harvested off shore.

While drying is a common way to arrest enzymatic activity, freeze drying is thought to be a gentle way of drying. The product is dehydrated by sublimation of the frozen water in the product and removal of the vapour. Because no liquid water is present during freeze drying, the product is locally dehydrated very rapidly, which reduces degradation due to enzymatic reactions.

1.4.2 Denaturation of autolytic enzymes

To completely avoid degradation of the raw material, enzymes in *C. finmarchicus* must be immediately inactivated. Heat is the most commonly used denaturing agent in food processing and preservation (Damodaran, 2008). From a nutritional standpoint, denaturation of proteins often improves the digestibility and biological availability of essential amino acids (Damodaran et al., 2008). Enzyme inhibitors and pH alteration are other methods to inactivate enzymes, but for nutritional purposes, a neutral pH would be preferred and inhibitors often have anti-nutritional effects or are toxic. Heat will primarily affect the stability of non-covalent interactions in proteins. Hydrogen bonding and electrostatic interactions are destabilised with increasing temperature, while hydrophobic interactions are stabilising, up to 70-80°C. When a sufficient amount of kinetic energy is applied, the protein unfolds, aggregates and precipitates as a function of decreased solubility. The denaturing temperatures of enzymes vary and depend, among other factors, on the amino acid composition and environmental factors (Damodaran et al., 2008).

1.5 Aims of the study

The main aim of the present work was to describe changes in the important biochemical components in *C. finmarchicus*, both in the live state in natural waters and in harvested *C. finmarchicus*. These changes are important to understand in order to obtain high value and high quality products from the biomass of *C. finmarchicus*.

Lipid contents and variability in individual *C. finmarchicus* in natural waters were studied from January to June in the Trondheimsfjord for three years (2009-2011) (Paper I). Individual copepods were compared with regard to fatty acid composition and size, and the fatty acid composition was compared with the fatty acid profile of the phytoplankton present. The size and fatty acid composition of copepodite V *C. finmarchicus* were also studied at different depths.

The content of astaxanthin in *C. finmarchicus* was studied during the spring of 2008 off the coast of Norway to obtain more information on the content, dynamics and distribution of astaxanthin and its esters (Paper II). A more detailed short-term study focusing on depth was undertaken inn late April 2009, in order to study the short-term variations in the content and esterification of astaxanthin.

Autolytic enzymes alter the biochemical composition of *C. finmarchicus* and lower the quality of the biomass post-harvest. Inactivation of autolytic enzymes was studied by freezing and drying the biomass, and heat was applied in order to denature autolytic enzymes. The biochemical content of the biomass and the enzymatic activity in the biomass of *C. finmarchicus* was studied following heat treatment (Paper III). Untreated

and heat-treated biomass of *C. finmarchicus* was stored at -20°C for 12 months, and the lipid and astaxanthin contents were studied after storage (Paper III). Freeze drying is regarded as a gentle process of drying and changes in the lipid class composition was studied by drying the biomass of *C. finmarchicus* under different conditions (Paper IV).

A method for the analysis of lipid class contents had to be established to study *post mortem* changes. The method used was TLC-FID, but some studies have expressed doubts using this method as a quantification tool. A control study of a method for the quantification of lipid classes using TLC-FID was therefore undertaken.

C. finmarchicus has been a part of a focus area at NTNU that has resulted in several PhD projects: Tokle (2006); Solgaard (2008); Overrein (2010); Tiller (2010); Bantle (2011) in addition to this thesis and the current PhD candidate Øystein Leiknes.

Chapter 2

Results and discussion

2.1 Lipid class analysis

In the present thesis, lipid classes were quantified by the use of thin layer chromatography coupled with a flame ionisation detector (TLC-FID). The technology was introduced in the 1970s, and is frequently used for quantification in research and in industry. Some authors have discussed problems associated with the quantification of lipid classes by the use of TLC-FID, and some limitations of the analysis technique have been suggested (Crane et al., 1983; Tvrzicka et al., 1990). The following section gives a brief introduction to the separation and quantification of the lipid classes that dominate in *C. finmarchicus* using TLC-FID.

2.1.1 Separation of lipid classes with TLC-FID

Lipid classes can be divided into polar and non-polar classes according to their polarity. The dominant non-polar lipid classes in *C. finmarchicus* are wax esters (WE) and triacylglycerides (TAG) (Lee and Hirota, 1973), and smaller amounts of free fatty acids (FFA) and cholesterol (CHO). The dominant polar lipid classes are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) (Farkas et al., 1988) and astaxanthin. Autolytic enzymes that are active *post mortem* could cause the formation of degradation products. Enzymatic activity on WE, TAG, PC, PE and astaxanthin could all lead to an increased content of FFA. Enzyme activity on WE could, in addition, result in an increased content of fatty alcohols, while TAG degradation could result in increased contents of the degradation products diacylglyceride (DAG) and monoacylglyceride (MAG). Degradation of phospholipids results in lyso-phospholipids and glycerophosphoryl backbones in addition to FFA.

The separation technique in TLC-FID is similar to conventional TLC because both techniques use silica as the separation material. Polar solvents are used to separate polar lipid classes and non-polar solvents to separate nonpolar lipid classes. The solvent system used to separate the non-polar lipids is hexane:methanol:formic acid (85:14:0.04, v:v:v) (Fraser et al., 1985); the lipid classes were separated as shown in Fig. 2.1A. The polar lipids were retained at the origin, and consisted of phospholipids (PL), MAG and astaxanthin; DAG co-chromatographed with CHO in this solvent system. The peak at the application point (origin) is sometimes used as a measure of phospholipids, but should rather be addressed as polar lipids.

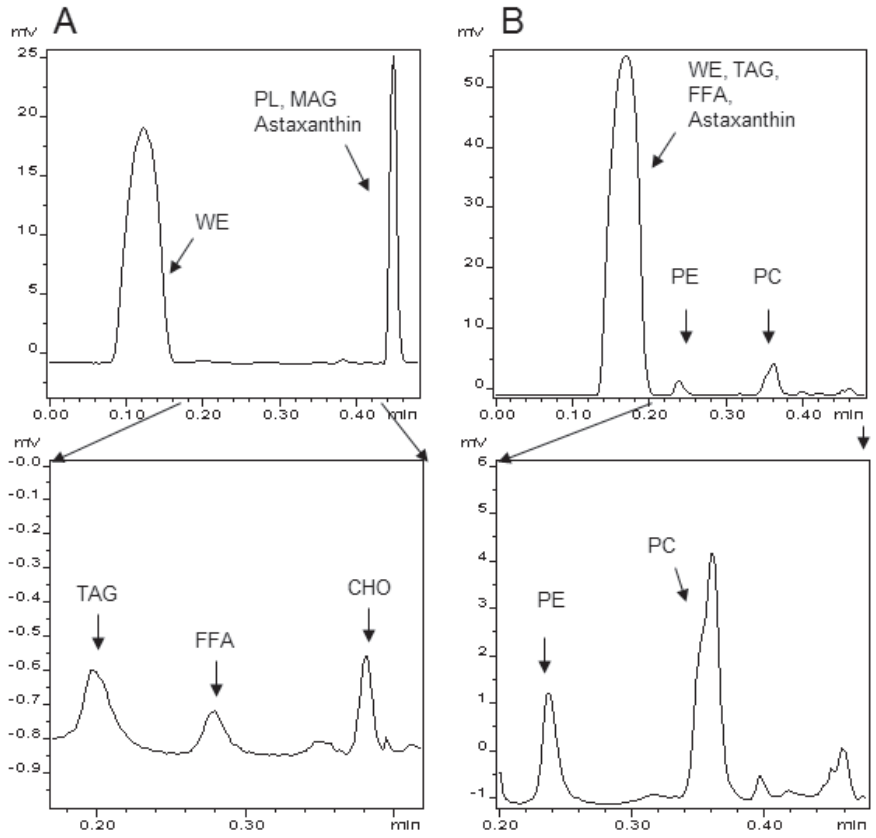

Figure 2.1: Chromatogram from *C. finmarchicus* obtained using TLC-FID. The non-polar development (A) separated the non-polar lipid classes, i.e. WE, TAG, FFA and CHO, and the polar development (B) separated the polar lipids, i.e. PC and PE. The x-axis shows the reverse retention time because the FID burns the rods in the opposite way compared to the development of the solvents. A detailed description of the method is given in Paper III.

The polar solvent used in the present study was chloroform:methanol:water (67:30:3, v:v:v), which allowed for separation of the main phospholipids in *C. finmarchicus* (Fig. 2.1B). The solvent front contained the non-polar lipid classes in addition to astaxanthin.

2.1.2 Quantification of lipid classes

Response factors (RF) are commonly used to quantify compounds in gas chromatography with FID, where RF is a calculation of the response of the compound of interest in relation to the response of an internal standard. The RF calculated in TLC-FID will vary depending on the amount of sample applied, which thereby complicates the quantification in TLC-FID (Crane et al., 1983). FID will respond to the amount of ionisable carbon in a compound; thus, challenges in the quantification of lipid classes are also caused by the very different fraction of ionisable carbon in each lipid class. As an example, PC contains

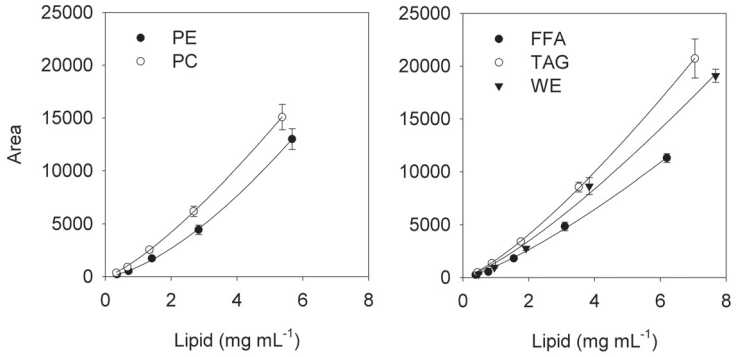

Figure 2.2: Examples of standard curves obtained with TLC-FID. Standard curves for PC and PE were obtained with polar development and WE, TAG and FFA were obtained with non-polar development. A detailed description of the method is given in Paper III.

about 60% (by weight) ionisable carbon and CHO contains 82% (Ackman, 1981), which will result in different responses in the detector for these components. Quantification can be achieved by the use of external standard curves for each lipid class (Ackman, 1981).

One of the challenges with using standard curves is the poor availability of standards of marine origin. Lipid classes of marine origin often have a high share of PUFA and one could expect a different response compared to standards with a low fraction of PUFA. However, Fraser et al. (1985) have shown by hydrogenating a TAG rich in PUFA and comparing the standard curves from both lipids that a reduction in the desaturation of the lipids did not have any significant effect on the response of TAG.

The length of the fatty acids could be of greater importance. Fraser and Taggart (1988) showed a deviation in the response between a natural TAG and triolein, a TAG with C18:1 n-9. A natural TAG has a range of fatty acids compared to the mono-isomeric triolein and will give broader peaks, and broader peaks have further been shown to result in a lower response compared to narrow peaks (Parrish and Ackman, 1983). Whitsett and Kennish (1988) also showed deviations in response between standards with different chain length within a lipid class. Natural standards would therefore be preferred over synthetic standards with a mono-isomeric fatty acid profile.

Furthermore, Miller et al. (1998) have shown that the use of sterylesters as standards for the determination of wax esters gave a higher response and resulted in an over-estimation of wax esters in *C. finmarchicus*. They suggested that wax esters in *C. finmarchicus* should be quantified with wax ester standards prepared from *C. finmarchicus*.

The standard curves used in the present thesis showed the best fit to polynomial regression ($y = ax^b$), except for WE which showed the best fit to linear regression. All compounds gave a squared correlation (r^2) of > 0.99 (Fig. 2.2). Some of the lipid classes showed the best fit to polynomial regression at low loadings and to linear regression at higher loadings (Volkman et al., 1986; Indrasena et al., 2007); this was found for TAG, FFA, PC and PE in the present study. WE showed the best fit to linear regression at all loadings. The response in the detector will vary among instruments and should be tested for each instrument.

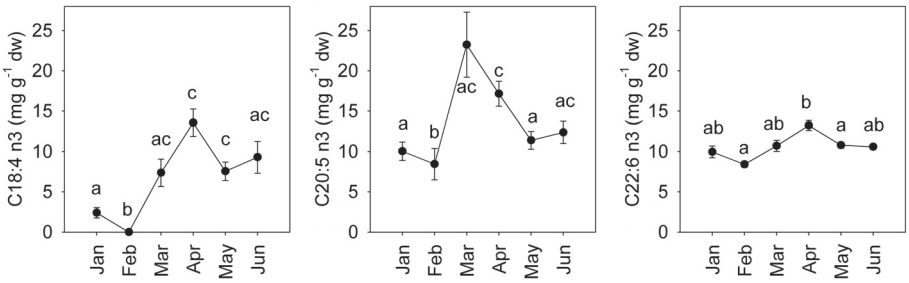

Figure 2.3: Average content of C18:4 n-3, C20:5 n-3 and C22:6 n-3 in *C. finmarchicus* (mg g^{-1}dw) from January to June (0-50 m) in 2009-2011 in the Trondheimsfjord. The statistics are described in Paper I.

The other challenge with standard curves is the precision of the work and was also recognized by Shantha (1992). The results from the present work showed that there were large differences between workers when applying the same amount of lipid onto the silica. It is therefore suggested that the standard curves and samples must be prepared by the same well-trained person. However, this step should be automated with an applicator to obtain more reproducible results and was also suggested by Shantha (1992). In addition, there are individual differences in the response of the rods and between lots (Farnworth et al., 1982). A solution could be to calibrate each rod or to use five rods as one calibration unit. The latter alternative was used in the present work.

In conclusion, TLC-FID can be a good tool for the quantification of lipid classes if precautions are taken to ensure a clean environment, precision in performing the work and with a thorough selection of standards.

2.2 Dynamics of lipids and astaxanthin

The content of lipids and astaxanthin in *C. finmarchicus* is dependent on the abundance and composition of the phytoplankton. The contents and variation of these compounds in *C. finmarchicus* was measured in samples collected of *C. finmarchicus* from natural waters in the early spring in Paper I and Paper II.

2.2.1 Lipid and fatty acid content in the early spring

Fatty acid content

The dominant n-3 PUFA in the phytoplankton were C18:4 n-3, C20:5 n-3 and C22:6 n-3 (Paper I). These fatty acids were also the dominant n-3 PUFA in *C. finmarchicus* (Paper I). The average content of C18:4 n-3 decreased from January to February, where after the fatty acid contents further increased towards June, and showed a maximum content of C18:4 n-3 in April (Fig. 2.3). The content of C20:5 n-3 also increased from January to June and showed a maximum content in March, where the increase from February to March was by 65%. The content of C22:6 n-3 was stable from January to June, and did

not show any pronounced variations. The content of C20:1 n-9 and C22:1 n-11 in CV of *C. finmarchicus* in surface waters did not change from February to June (Paper I).

The variations in the dominant n-3 PUFA and in the two main monounsaturated fatty acids (MUFA) C20:1 n-9 and C22:1 n-11 found in *C. finmarchicus* from several studies are listed in Table 2.1. The content of C18:4 n-3 can range from 0% to 22 % of the TFA of total lipids, while the fractions of C20:5 n-3 and C22:6 n-3 were found to be from 6% to 22% and from 7% to 33 % of TFA, respectively. The variability in these n-3 PUFA is considerable and reflects the diet.

The fatty acid composition of *C. finmarchicus* was also studied through the upper 30 m in a short-term study in 2009 off the coast of central Norway. The main aim of the short-term study was to describe the content of astaxanthin; this work is presented in Paper II. The fatty acid composition of the samples is presented in Fig. 2.4. The samples were taken from the same location over a period of 30 h. During the study there was a bloom of *Phaeocystis pouchetii*, which has been shown to contain the fatty acid C18:4 n-3 (Hamm et al., 2001). Fig. 2.4 shows that the content of C18:4 n-3 in *C. finmarchicus* was highly variable throughout the study, while the content of C20:5 n-3 and C22:6 n-3 was stable. The content of C18:4 n-3 was correlated with the total lipid content in the biomass (Spearman correlation, $r_s = 0.91$, $P<0.001$). The variability of C18:4 n-3 therefore explained much of the variability in the lipid content.

The content of C18:4 n-3 and C16:1 n-7 can be used as trophic markers for dinoflagellates and diatoms respectively (Graeve et al., 1994). The use of C18:4 n-3 as a trophic marker is also illustrated by the variation of this fatty acids in the short-term study (Fig. 2.4). The content of C18:4 n-3 found in the last sample varied from 8 mg g^{-1} dw at a depth of 30 m to 14 mg g^{-1} dw at a depth of 15 m, representing a difference of 43%. These results illustrate the high variability of fatty acids, not only over time, but also as a consequence of depth.

Dry weight and lipid content

C. finmarchicus was found to ascend to the surface from January to March in the Trondheimsfjord (Paper I). The dry weight and total fatty acids (TFA) of individual *C. finmarchicus* decreased from January to February, and thereafter increased from February to June. Both the dry weight and TFA per individual had reached the same content in June as in January.

C. finmarchicus was, in general, smaller and leaner at the surface and the dry weight, body volume, TFA and volume of the oil sac increased towards deeper waters. The dry weight and content of total fatty acids per individual CV of *C. finmarchicus* showed a high correlation (Spearman correlation, $r_s = 0.87$). *C. finmarchicus* started to descend to deeper waters in May, which was also shown by the increase in C18:4 n-3, C20:5 n-3 and C22:6 n-3 in deeper waters.

CV migrated towards the surface in January and February (Paper I). These CV probably use their lipid reserves to mature to females, as Gatten et al. (1980) showed a reduction of 50% in the lipid reserves when *C. helgolandicus* matured to females. Some of the CV did not mature to females, possibly caused by a shortage of lipid reserves, and the dry weight and lipid content decreased from January to February in these CV. The decrease was probably caused by the lack of food in the surface waters, also suggested by Sargent and Falk-Petersen (1988). Other authors have also found a decrease in size and

Table 2.1: Content of n-3 PUFA (C18:4 n-3, C20:5 n-3 and C22:6 n-3), C20:1 n-9 and C22:1 n-11 in the total lipid of *C. finmarchicus* sampled from natural waters. Rounded numbers express % of total fatty acids (weight).

Stage	Location	Time of year	C18:4 n-3	C20:5 n-3	C22:6 n-3	C20:1 n-9	C22:1 n-11	Reference
CIV-CV	Arctic, North Sea	n.r.	11-16	12-18	9-14	3-10	5-9	Kattner (1989)
CIV-F	Arctic	June-July	2-20	7-22	8-16	5-13	5-16	Kattner et al. (1989)
CIV-F	North Sea	All year	0.4-13	11-22	10-33	1-7	1-12	Kattner and Krause (1989)
CV	Arctic	June	22	13	10	7	9	Graeve et al. (1994)
CIV-CVI	Northern Norway	April-July	n.r.	15-21	21-31	n.r.	n.r.	Evjemo et al. (2003)
CIII-CVI	Coast off Norway	Spring-autumn	11-19	10-16	12-19	2-10	4-17	Overrein (2010)
CV	Northwestern Atlantic	Late autumn	11	12	7	5	8	Pepin et al. (2011)
CV-F	North Atlantic	April	3	18	13	21	9	Teerawanichpan and Qiu (2011)
CV	Scotland	All year	0.4-9	5-13	2-13	3-7	8-16	Clark et al. (2012)
CV	Central Norway	Jan-June	0-13	6-22	8-17	3-6	5-12	Paper I

Abbreviations: Not reported (n.r.), Copepodite stage III-V (CIII-CV), Female (F)

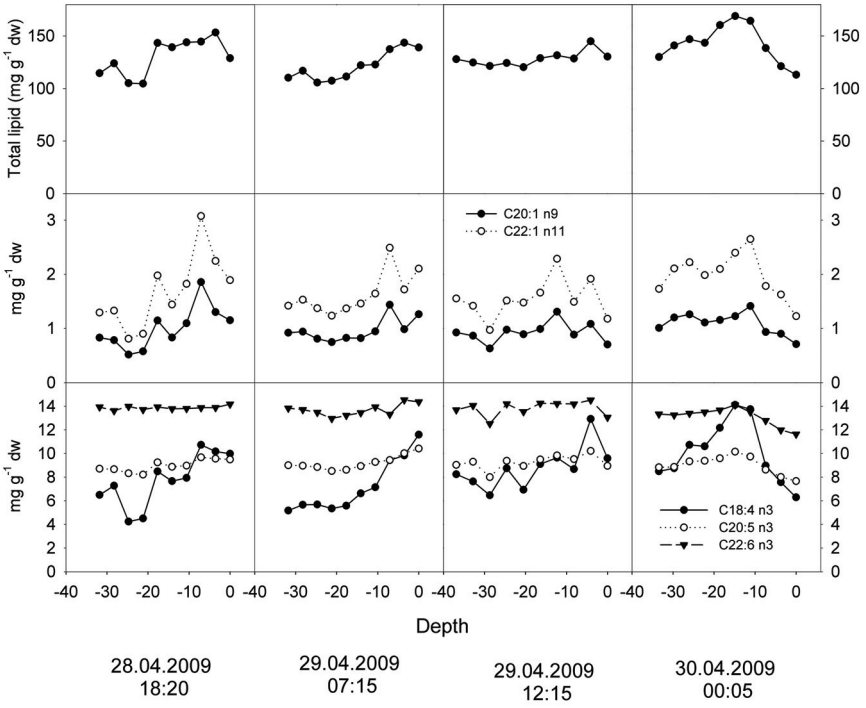

Figure 2.4: Fatty acid content of the dominant n-3 PUFA, C20:1 n-9 and C22:1 n-11 and the content of total lipid in *C. finmarchicus* at a fixed location off the coast of central Norway. The details of the location and sampling technique are given in Paper IV.

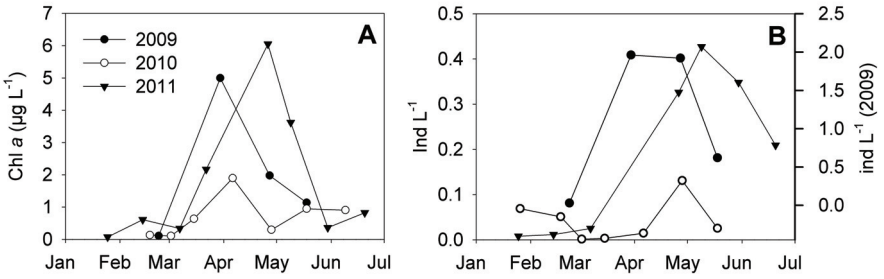

Figure 2.5: Concentration of chl *a* (g L^{-1}) and *C. finmarchicus* (ind L^{-1}, all development stages) from January to June in 2009-2011.

lipid (Kattner and Krause, 1987; Jonasdottir, 1999; Miller et al., 2000; Pasternak et al., 2001) in the winter. Miller et al. (1998) also found that large CV in the surface waters in December moulted to females while the smaller C5 were left at the surface waiting for food until March. In March they found larger C5 which they attributed to be the new generation C5.

Several theories regarding what triggers the onset of migration have been suggested; an increase in lipids is one of them (Irigoien, 2004). The numbers of CV in intermediate and deep waters started to increase in May, following the phytoplankton bloom. These specimens were larger and contained more lipids compared to CV at the surface (Paper I). The fatty acid composition of the copepods in deeper waters resembled the dominate fatty acid of the phytoplankton, suggesting that these CV had been feeding on the phytoplankton bloom and further descended for dormancy. The number of females increased at the surface in May. We hypothesise that CV with high lipid contents will descend for dormancy, while CV with a lower content of lipids after the phytoplankton bloom will moult to females and start a new generation of *C. finmarchicus*. The results from our study suggest that lipid accumulation could have a strong connection with the onset of dormancy. Johnson et al. (2008) did not find an environmental factor that explained the onset of dormancy in *C. finmarchicus*, and also suggested that lipid accumulation could be an explanation.

In the spring, the maximum concentration of phytoplankton and the maximum numbers of *C. finmarchicus* were highly variable between the sampling periods (2009-2011) in the Trondheimsfjord (Paper I). The maximum concentration of chlorophyll *a* (chl *a*) and the maximum numbers of *C. finmarchicus* were considerably lower in 2010 compared to 2009 and 2011 (Fig. 2.5).

Lipid metabolism

C20:1 n-9 and C22:1 n-11 are fatty acids that are not a part of the diet and are synthesised *de novo*. Furthermore, these fatty acids have been suggested to be converted to fatty alcohols by a fatty acyl co-enzyme A oxido-reductase (Pascal and Ackman, 1976; Sargent and Henderson, 1986); three reductases have been identified (Teerawanichpan and Qiu, 2011). The synthesis of wax esters involves the esterification of one molecule of

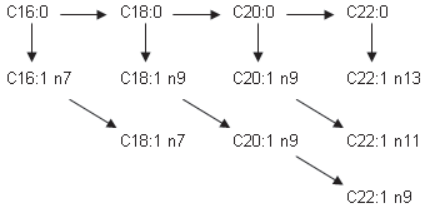

Figure 2.6: Proposed bio synthetic route of fatty acids in *C. finmarchicus* (adapted from Sargent and Henderson, 1986).

fatty acid with one molecule of fatty alcohol. The formation of fatty alcohols has been suggested to be the rate-limiting step of the synthesis of wax esters (Lee et al., 1972; Pascal and Ackman, 1976). *C. finmarchicus* synthesises fatty alcohols during the phase of lipid accumulation. The content of the fatty acids C20:1 n-9 and C22:1 n-11 (Paper I) did not increase in CV of *C. finmarchicus* during the lipid accumulation phase, suggesting that they were converted to fatty alcohols.

The content of C14:0 and C16:0 did not increase, even though these fatty acids were dominant in the phytoplankton that were present (Paper I). These fatty acids may serve as energy sources through catabolism, they could be converted to the corresponding fatty alcohol or they could serve as a precursor for further elongation and desaturation to yield C20:1 n-9 and C22:1 n-11 and finally be converted to fatty alcohols. The proposed *de novo* bio-synthetic route for fatty acids is shown in Fig. 2.6.

Several authors have suggested that *C. finmarchicus* can use fatty acids in the diet in their synthesis of fatty alcohols (Lee et al., 1972; Sargent and Henderson, 1986; Sargent and Falk-Petersen, 1988; Kattner and Krause, 1989; Graeve et al., 2005), but it has also been suggested that proteins and carbohydrates are more likely to serve as precursors for the *de novo* synthesis of fatty alcohols (Sargent and Henderson, 1986). *C. finmarchicus* can perhaps switch to *de novo* synthesis of fatty acids depending on the fatty acids that are present in the diet (Sargent and Falk-Petersen, 1988), and selectively accumulate and retain fatty acids in accordance to its metabolic requirements (Graeve et al., 2005). The results from Paper I suggested a substantial use of dietary fatty acids as precursors for further synthesis of fatty alcohols, while the longer and more unsaturated fatty acids C18:4 n-3, C20:5 n-3 and C22:6 n-3 were retained in *C. finmarchicus*. C20:5 n-3 and C22:6 n-3 are needed in phospholipids, while n-3 PUFA helps to fluidise the long-chain monounsaturated fatty alcohols in wax esters (Sargent and Henderson, 1986). Wax esters need to have a low melting point in order to remain fluid at low temperatures. However, Pond and Tarling (2011)have suggested that wax esters with high levels of unsaturation will solidify at depth over 500 m. The lower buoyancy of the copepod would contribute to neutral buoyancy with the water through dormancy.

The content of C22:6 n-3 in the WE of *C. finmarchicus* has been shown to be low (0% to 5%) (Table 1.1), while the content of C22:6 n-3 is around 40% of phospholipids (PL) (Table 1.2). However, the content of C22:6 n-3 in the total lipid of *C. finmarchicus* can comprise up to 33% of TFA (Table 2.1), and the fraction of C22:6 n-3 in PL cannot account for the entire amount found in total lipids. Overrein (2010) recognised this phenomenon and suggested that increased contents of C22:6 n-3 could be incorporated in the PL of *C.*

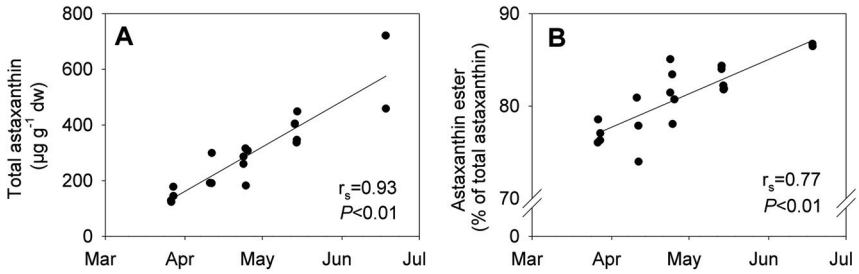

Figure 2.7: The correlation of the content of astaxanthin (μg g-1 dw) (A) and the fraction of astaxanthin esters (% of total astaxanthin) (B) over time, in samples of *C. finmarchicus* from March to June in 2008; the data are presented in Paper II. The Spearman correlation coefficient (r_S) were 0.93 and 0.77, respectively and $P < 0.01$ at both correlations (n=19).

finmarchicus as she found a small increase in PL in the spring. An increased content of PL could be found in female *C. finmarchicus* that produce eggs rich in PL (Ohman and Runge, 1994). The quantitative content of PL in females of *C. finmarchicus* with eggs could therefore be higher compared to that in CV.

To conclude, the abundance of *C. finmarchicus* and its content of n-3 PUFA are on average high from April to June in the Trondheimsfjord, but the fractions of C18:4 n-3, C20:5 n-3 and C22:6n-3 still vary considerably during harvesting of *C. finmarchicus*.The abundance of *C. finmarchicus* was highly variable at the surface between years and could be the case in other fjords as well. Harvesting of a small population would not be sustainable.

2.2.2 Astaxanthin content in the early spring

Copepods cannot synthesise astaxanthin *de novo* and needs to be supplied with carotenoids in the diet. The main precursor for astaxanthin synthesis is β-carotene. Most phytoplankton species contain β-carotene in addition to other carotenoids (Jeffrey and Vesk, 1997). In samples of *C. finmarchicus* collected off the coast of Norway (Paper II), the content of astaxanthin increased from March to June (Fig. 2.7). The content of astaxanthin showed a positive correlation with time (Spearman correlation, r_s=0.93, P<0.001). Most of the astaxanthin was esterified to one or two fatty acids (Foss et al., 1987, Paper II), and the fraction of astaxanthin esters increased from 77% of total astaxanthin to 87% during the same time period. An increased content of astaxanthin in the spring has to our knowledge, not been reported before. During the short-term study (30 h) at a fixed location in the early spring (Paper II), the total content of astaxanthin remained constant, but the fraction of free astaxanthin was reduced by 50%.

The content of astaxanthin showed pronounced variability from day to day during the study from March to June, suggesting a highly dynamic content. The same variability has been described for *Acartia* sp. (Kleppel et al., 1985; Andersson et al., 2003; Van Nieuwerburgh et al., 2004; Holeton et al., 2009), *Calanus pacificus* (Juhl et al., 1996) and *Calanus helgolandicus* (Sommer et al., 2006). In *C. helgolandicus,* Sommer et al. (2006) showed an increase of 60% in the astaxanthin content at night compared to during the day, and

both the contents of free- and esterified astaxanthin increased. Some authors have found higher feeding activity in copepods at night (Kleppel et al., 1985; Andersson et al., 2003; Sommer et al., 2006) and a correspondingly higher content of astaxanthin (Juhl et al., 1996). However, Holeton et al. (2009) found that increased food availability resulted in higher accumulation of astaxanthin only up to food concentrations of 150 μg C L^{-1} for *Acartia bifilosa*. At higher food concentrations, the astaxanthin content decreased. The daily variations in the content of total astaxanthin could be the result of variations in the feeding activity of *C. finmarchicus*.

The accumulation of astaxanthin in the spring (Paper II) coincided with the accumulation of energy reserves in the form of wax esters. When *C. finmarchicus* is abundant, the copepod resides at the surface during the day (Cushing, 1951; Durbin et al., 1995). Astaxanthin serves as a light quencher and protects copepods against harmful UV radiation, and wax esters with a high degree of PUFA also need protection. Astaxanthin could then serve as extra protection for the copepods which could partially explain the increased content in the spring. The copepods would also need protection for the increasing amount of sun during the spring. However, photo-protection alone cannot explain the rapid changes in both the colour and content of astaxanthin in *C. finmarchicus*.

Many suggestions have been made for the biological role of astaxanthin, but it is difficult to make conclusions because studies from natural marine waters are scarce. Van Nieuwerburgh et al. (2004) suggested that the content of esterified astaxanthin increases during growth and biomass accumulation in copepods, and that their pool of free astaxanthin is reduced when there is no further need for the pigment e.g. for photoprotection. Furthermore, Lotocka et al. (2004) have hypothesised that the physiological replacement of oxygen in the copepod tissue by astaxanthin that may serve as acceptors of free electrons released during the rapid combustion of lipids, while Ringelberg (1981) has suggested that carotenoids may function as metabolic fuel for copepods in addition to possible other functions. Vitamin A and other retinoids appear to be absent in several copepod species, including *C. finmarchicus* (Marshall and Orr, 1972; Rønnestad et al., 1998; Moren et al., 2005). In Atlantic salmon and crustaceans, astaxanthin has been shown to function as pro-vitamin A (Christiansen et al., 1994; Linan-Cabello et al., 2002). It is likely that the carotenoids can serve multiple biochemical functions in copepods, but more research is needed in order to elucidate their different metabolic roles.

Free astaxanthin is a non-polar molecule and can be located in cell membranes (Gruszecki, 2010). However, free astaxanthin is often found to form aggregates caused by the polar groups at the end of the molecule (Fig 1.2). These aggregates will shift the absorption spectra of carotenoids and can further lead to a lower antioxidant capacity (Edge and Truscott, 2010). By esterifying these polar groups with long non-polar fatty acids the astaxanthin molecule probably forms aggregates less readily. The fact that most of the astaxanthin is found in an esterified form could be a strategy to avoid astaxanthin aggregation and further lower antioxidant capacity. The antioxidant capacity of astaxanthin esters have been shown to be high in relatively non-polar solvents, so astaxanthin could therefore be a well-functioning antioxidant in non-polar lipid membranes. The oxygen quenching activity (antioxidant capacity) of free- and esterified astaxanthin was measured by Kobayashi and Sakamoto (1999). They found that the antioxidant capacity of astaxanthin esters was similar or even higher compared to free astaxanthin in relatively non-polar environments, but the opposite was found for polar environments. Kobayashi

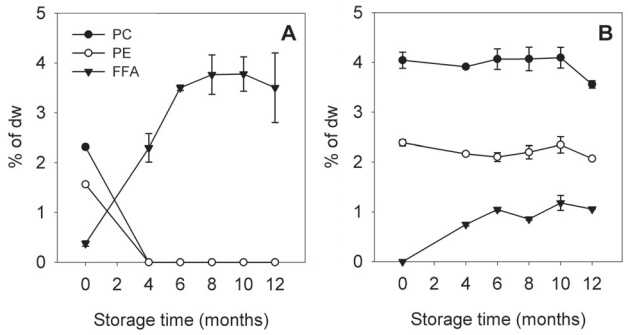

Figure 2.8: Storage of untreated biomass of *C. finmarchicus* (A) at -20°C. Storage of heat treated biomass of *C. finmarchicus* (70°C, 15 min) (B) at -20°C (detailed information is given in Paper III).

and Sakamoto (1999) suggested an activation mechanism of astaxanthin in different parts of the tissue, where the esterification of astaxanthin would maintain the oxygen quenching ability of the molecule when it is subjected to relatively non-polar environments as in cell membranes.

In conclusion, the content of astaxanthin can change considerably from day to day during harvesting of *C. finmarchicus*. The content can also increase from the beginning of the harvesting period in April compared to in June. Astaxanthin probably functions as a photo-protectant in copepods, but it could have other functions as well.

2.3 *Post mortem* changes in *C. finmarchicus*

The digestive enzymes in *C. finmarchicus* are active *post mortem,* resulting in changes in the lipid and protein composition (Overrein, 2010). Several methods can be used to avoid or reduce the enzymatic activity in the biomass of *C. finmarchicus*. Temporary inactivation of autolytic enzymes in *C. finmarchicus* was studied in Paper III and IV. Denaturation of autolytic enzymes in *C. finmarchicus* was studied in Paper III. Heat treatment is a common way to inactivate enzymatic activity by denaturation of the protein structure (Damodaran, 2008).

2.3.1 Temporary inactivation of autolytic enzymes

Frozen storage

Untreated biomass was stored at -20°C for 12 months (Paper III). All phospholipids (PL) were lost after 4 months of storage, while the content of free fatty acids increased. The content of astaxanthin was stable in the biomass during storage, and no reduction was found after 12 months at -20°C. The fraction of n-3 fatty acids was also stable throughout 12 months of storage (Fig. 2.8).

Lipase activity in fish muscle has long been recognised as a challenge during cold storage. Lovern and Olley (1962) found the highest accumulation of free fatty acids in

fish muscle at -4°C, and activity has been observed even at -70°C (Brockerhoff, 1974). Overrein (2010) found a small increase in the level of FFA and a small decrease in the content of PL in *C. finmarchicus* stored at 0°C for 24 h. Similar findings have been reported for krill (Kolakowski and Sikorski, 2000), *Calanus pacificus* (Ohman, 1996) and *Artemia* (Sasaki and Capuzzo, 1984) that had been stored at -15°C or -20°C.

PL appears to be more susceptible to degradation by enzymatic activity compared to WE. The content of WE was stable during storage (Paper III), and this has also been shown by others (Ellingsen, 1982; Saether et al., 1986; Overrein, 2010). Kolakowski and Sikorski (2000) suggest that PL are degraded first because the activity of phospholipases is higher compared to that of other lipases. This could also be caused by better contact between the enzyme to the more hydrophilic phospholipids, as suggested by Ellingsen (1982). WE are physically separated in an oil sac in *C. finmarchicus*, resulting poor enzyme to substrate contact. The hydrophobic character of WE would also provide poor enzyme to substrate contact.

The content of astaxanthin and the fraction of n-3 PUFA was maintained during storage and could imply an overall oxidatively stable biomass, despite the increased content of FFA. An increased content of FFA has been found to reduce the oxidative stability, cause off flavours as well as foaming, and is regarded as a parameter for poor quality (McClements and Decker, 2008).

The results show that PL in the biomass of *C. finmarchicus* was lost before 4 months of storage at -20°C. To store untreated biomass and avoid phospholipid degradation caused by enzymatic activity, storage must occur at a temperature of -70°C.

Plate freezing

Plate freezing is a common way to freeze large amounts of biomass on fishing vessels. As discussed, the biomass of *C. finmarchicus* has a high enzymatic activity that results in the degradation of phospholipids (Paper III and IV). The core of thick plates needs a longer freezing time compared to thinner plates, and could result in increased degradation of phospholipids. The freezing time was measured in biomass of *C. finmarchicus* frozen at different thicknesses ranging from 2.5 cm to 13.5 cm (Fig. 2.9). The core of the thickest plate reached -20°C 5 hours after the thinnest plate. The ash content ranged from 8-14% of dry weight, and the addition was believed to be a result of variable salt content from the sea water. When the biomass is frozen in plates on the vessel directly after trawling, a substantial amount of sea water is frozen with the biomass. The content of total lipid in the core of frozen plates showed pronounced variations (Fig. 2.9). The increased content of total lipids was partly explained by a lower ash content in the core of the 5 and 7.5 cm thick plates compared to the 2.5, 10 and 13.5 cm thick plates. The content of the main phospholipids, PE and PC (Paper III and IV), in the biomass showed minor variations.

The difference in freezing time from the thinnest to the thickest plate was 5 hours and did not result in any significant difference in degradation of phospholipids. Overrein (2010) only found a small decrease in phospholipids when the biomass was stored at 0°C for 24 hours. The content of phospholipids with variable plate thickness showed minor variability, which also suggests that the freezing time does not have a significant impact on the enzymatic activity and degradation of phospholipids.

The ash content of *C. finmarchicus* has been shown to be 3.6% of dw (Marshall and Orr, 1955). The plates in Fig. 2.9 show an ash content of 8% to 14% of dw. The additional

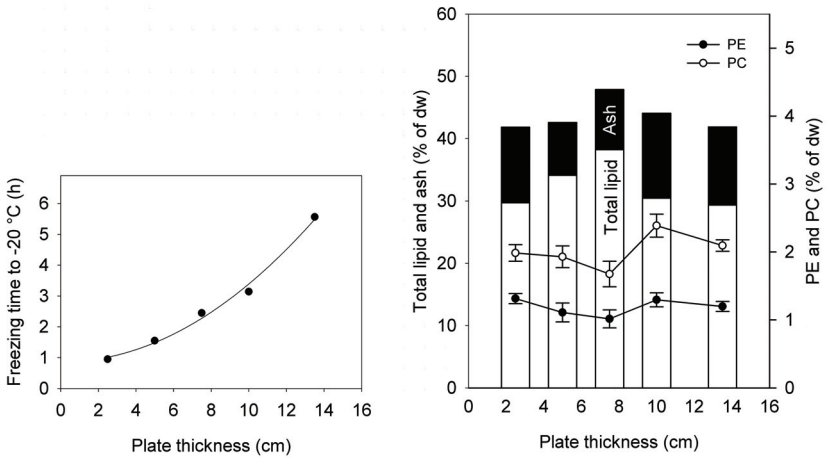

Figure 2.9: The biomass of *C. finmarchicus* was frozen in plates with different thicknesses. The middle of the plate (1x1 cm) was cut out and the biomass was analysed for total lipid, ash, phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) (% dw). Two plates of each thickness were analysed and is presented as mean ± SD. The x-axis shows the thickness of the plate. The left graph shows the time needed for the core of the plate to reach -20°C.

ash content was probably salt, which would result in a variable salt content of 4.4% to 10.4 % of dw, a difference of 57%.

Samples taken from the bottom, the top and the middle of three plates showed an increased content of ash in the middle of the plate (Fig. 2.10). Studies on freezing soil (Harris, 1995) have shown that solutes will follow the freezing front and concentrate in bands with high salt content. During freezing, pure ice separates first and other solutes will be more concentrated and transported with the freezing front. The least soluble solute will then precipitate, which could be salts (Scopes, 1994). Enzymes could be concentrated as the salts are crystallised, and could lead to areas with higher degradation. This could further lead to a decrease in storage time. Another effect of the precipitation of salt is a change in pH as the buffer capacity becomes reduced.

A freezing temperature of -25°C instead of -15°C ha been suggested to minimise denaturation because of the advantage of solidification of the material (Scopes, 1994).

To conclude, the thickness of the plate has a limited effect on the degradation of phospholipids. However, the plate thickness results in a variable content of lipids and salt in the biomass of *C. finmarchicus*. An increased salt content could have some effect on the activity of enzymes, which could further lead to a variable quality of the biomass.

Freeze drying

Different freeze drying processes (vacuum, atmospheric and nitrogen) were evaluated as preservation methods for *Calanus finmarchicus* biomass in Paper IV. Atmospheric freeze drying (AFD) gave the highest drying rate and nitrogen freeze drying (NFD) gave the lowest drying rate (Table 2.2). The content of phospholipids (PL) was reduced by 57% in

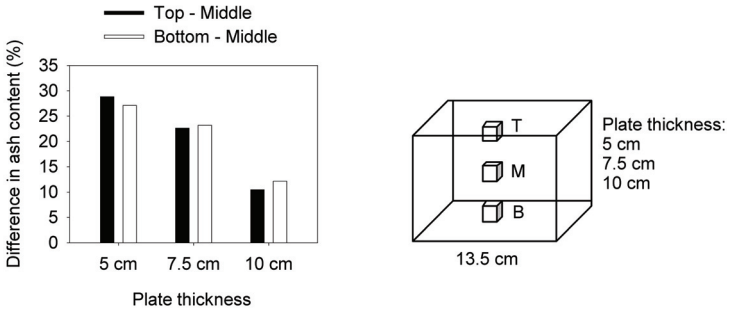

Figure 2.10: Difference in the ash content (%) in relation to plate thickness. The difference was calculated by subtracting the ash content of the top of the plate with the ash content in the middle of the plate, and between the contents on the bottom of the plate and the middle. The samples were taken from the plate as illustrated on the right (T=Top, M=Middle, B=Bottom).

AFD and VFD compared to the content in the raw biomass. 92% of the PL was lost in NFD. The reduction in PL resulted in an increase in free fatty acids (FFA), whereas AFD resulted in the lowest content of FFA. NFD resulted in the highest content of FFA whereas FFA comprised 32% of the total lipid (Table 2.2). The content of secondary oxidation products was higher for NFD compared to AFD and VFD. A product is considered rancid when the TBARS value is higher than 1 (Østerlie et al., 1999); NFD was the only biomass considered rancid according to this criteria.

As discussed above, the lipids in *C. finmarchicus* are rapidly degraded *post mortem* due to highly active autolytic enzymes. This results in a lower content of PL and increased content of FFA (Overrein, 2010). Lipases responsible for detaching fatty acids from PL are also active under frozen conditions, even at -70°C (Brockerhoff, 1974). In the present study, the biomass was kept at -10°C for 24 h before grinding. Grinding before drying may have resulted in the liberation of enzymes from the tissue, leading to better enzyme-substrate contact and hence more degradation of PL. Saether et al. (1986) found greater phospholipid degradation during storage of homogenised krill (*Meganyctiphanes norvegica*) compared to whole krill, but found no similar differences in samples of another krill species, *Thysanoessa* sp.

AFD took 24 h to obtain a dry product while NFD took 5-6 days to obtain a stable product. The time elapsed could be the most important factor for the degradation of PL. The enzymes were active over a longer period of time, which may have resulted in a greater degradation. The rancidity found in the dried product with NFD may have been caused by the higher content of FFA in the biomass, because FFA increase the oxidation potential (McClements and Decker, 2008).

The biomass of *C. finmarchicus* could have enzymatic activity at low water contents, such as in dried biomass (Parkin, 2008). If the activities are arrested after drying, they will be restored again when the biomass is re-hydrated (Bantle et al., 2009).

To conclude, AFD resulted in the best quality of biomass of dried *C. finmarchicus* compared to VFD, and NFD is not suited for maintaining high quality of biomass. The content of phospholipids was also found to decrease with AFD, which could have been caused by the pre-treatment of biomass before drying. For the best quality of dried

Table 2.2: Drying temperature, drying rate, final moisture content, phospholipids (PL), free fatty acids (FFA), n-3 PUFA and secondary oxidation products (TBARS) in the biomass of *C. finmarchicus* freeze dried with different drying processes.

Freeze drying technique	Atmospheric AFD	Vacuum VFD	Nitrogen NFD
Drying temperature (°C)	-6	-17	-8
Moisture (% ww)	13	8	20
Drying time until stable weight (h)	24	48-60	120-140
Drying rate ($kg_{H_2O}kg^{-1}{}_{dw}h^{-1}$)	0.22	0.10	0.04
PL (mg g^{-1} dw)	17±5	16±8	1
FFA (mg g^{-1} dw)	17±4	29±15	47±10
n-3 PUFA (mg g^{-1} dw)	43±1	41±1	42±1
TBARS (μmol g^{-1} lipid)	0.7±0.3	0.5±0.1	1.5±0.4

biomass of *C. finmarchicus* the best alternative would be to inactivate the enzymes prior to drying, for example with heat (Paper III).

2.3.2 Denaturation of autolytic enzymes

Biomass of *C. finmarchicus* was heat treated at 70°C to inactivate autolytic enzymes (Paper III). An instant temperature increase to 70°C was obtained by mixing with boiling fresh water. The activity of selected autolytic enzymes was measured before and after heat treatment, and the results show that 5 min of heating was enough to inactivate the enzymes (Fig. 2.11). The combination of heat treatment and frozen storage was also studied. Storage of heat treated biomass (70°C, 15 min) at -20°C for 12 months showed a stable content of PL and a low content of FFA (Paper III) (Fig. 2.8B). The content of astaxanthin and fraction of n-3 PUFA remained constant during the storage, demonstrating that these components are oxidatively stable in biomass.

Most of the total lipid was retained in the biomass during heating and no changes were found in the content of phospholipids and the fraction of n-3 fatty acids. The crude protein remained unchanged. Water soluble components were released to the water and gave an average reduction of 15% of dry weight in the biomass. The ash content per dw of the biomass was reduced by 50%.

The optimum temperature for proteases in extracts of *C. finmarchicus* was found to be 50°C (Solgaard et al., 2007) and was similar as for krill (Kolakowski and Sikorski, 2000; Osnes and Mohr, 1985). Overrein (2010) found an increased content of free amino acids when heating biomass of *C. finmarchicus* to 40°C, 50°C and 60°C. The biomass also had an increased content of free amino acids when the biomass was heated to 70°C, but this was probably caused by the 7 min of heating time from 0-70°C (Overrein, 2010). Krill proteases were inactivated between 63-80 °C, but some tyrosinase activity was still detected, even after 15 min of boiling (Suzuki, 1981). The instant temperature increase to 70°C (Paper III) was sufficient for inactivation of proteases. The thermal stability of krill lipases was found to be relatively low and the enzymes were inactivated at 60°C (Kolakowski and Sikorski, 2000). Overrein (2010) found a decreased content of PL when heated to 40°C, 50°C and 60°C, indicating lipase activity, but this could have been caused by the prolonged heating time from 0°C to the desired temperature.

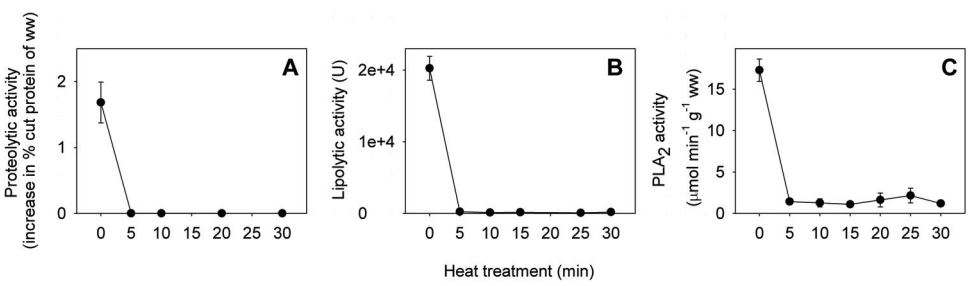

Figure 2.11: Proteolytic activity (increase in the amount of degraded protein in % wet weight) (A), lipolytic activity (increase in fluorescence) (B) and phospholipase activity (mol $min^{-1}g^{-1}$ ww) (C) in heat treated *C. finmarchicus* (detailed information is given in Paper III).

Autolytic enzymes will degrade PL to FFA in the harvested biomass of *C. finmarchicus* if special care is not taken. Inactivation of enzymes leads to the stabilisation of PL in the biomass. In addition, the specified heat treatment will provide a biomass that can be stored for at least one year at -20°C without any changes in the lipid content.

2.4 *C. finmarchicus* - a challenging raw material

The n-3 fatty acid contents in *C. finmarchicus* will vary considerably depending on both the quantity and quality of the phytoplankton present in spring and summer (Paper I). The fatty acid 20:5 n-3 will be most dominant during the bloom of diatoms, and the content of 18:4 n-3 and 22:6 n-3 will increase later in spring caused by a bloom of dinoflagellates and other flagellates (e.g. *Phaeocystis pouchetii*). The fatty acids 18:4 n-3 and 20:5 n-3 will dominate the lipid content. In the spring the wax ester contents in *C. finmarchicus* will increase, indirectly shown as an increase of total fatty acids in the present work.

Our study also shows that the astaxanthin content will vary considerably both within the season as well as from day to day (Paper II). The variations from day to day could be tremendous. We found e.g. a reduction of 40% from one day to another in June 2008 and are in accordance to Sommer et al. (2006) that found 60% higher content at night samples compared to day in *C. helgolandicus*.

The content and composition of lipids and astaxanthin may be altered post-harvest due to high enzyme activity. Overrein (2010) has shown a high degradation of phospholipids in *C. finmarchicus post mortem*. This is in agreement with our study demonstrating that the content of phospholipids was not stable during storage and will be a substrate for enzymatic activity yielding free fatty acids (Paper III). The biomass should preferably be stabilised on board of the vessel directly after harvest, e.g. by heating, to maintain the initial phospholipid contents. Heating at 70 °C for 15 min was sufficient to maintain the phospholipids through one year of storage at -20 °C (Paper III). Wax esters on the other hand have shown to be stable *post mortem* and during storage without stabilisation.

Even though the astaxanthin content in harvested biomass of *C. finmarchicus* will be highly variable, the astaxanthin content have been shown to be stable post-harvest.

Results from Paper III showed a stable astaxanthin content in biomass of *C. finmarchicus* stored for one year at -20 °C.

The variability of lipids and astaxanthin in live specimens will result in a raw material with a highly variable content of these components. Due to a short time-window of harvesting (spring and summer) the content of lipids and astaxanthin in the biomass will vary during this period and a standardisation of the raw material will be difficult. There is a need for a standardised content of lipids and astaxanthin in feeds for the salmonid aquaculture (Bogevik, 2011). A standardisation can perhaps be done before or after processing the biomass.

The abundance of *C. finmarchicus* in surface waters can be highly variable between years in the Trondheimsfjord (Paper I), which may be the case also in other fjords. The concentrations of *C. finmarchicus* can be high in surface waters when the phytoplankton or other potential food particles is present in the spring or summer. This short time-window is the most likely period for commercial harvesting of high quality *C. finmarchicus*. In Paper I we suggest that CV will start the vertical migration when a significant lipid level has been reached. CV is observed in deeper waters during May in the Trondheimsfjord and a possible harvest should exploit *C. finmarchicus* in the period when it is most abundant and before the majority of the copepods descend to depth.

When this copepod is present in low numbers in a fjord, harvesting can have some impact on both the remaining population and the predators of the zooplankton. Harvesting on a low population would maybe not be sustainable. However when *C. finmarchicus* is abundant, harvesting could even be a positive input for the population, leaving more food for the remaining stock.

Our results suggest that fjords are not a suited harvesting arena for *C. finmarchicus*, at least not when the abundance is low. Harvesting of *C. finmarchicus* must be sustainable in order to exploit this resource and ecological models could be a sufficient method to predict the outcomes of the remaining population after harvesting.

Chapter 3

Conclusion and future work

3.1 Conclusion

In the future, biomass of *C. finmarchicus* could be a new source of marine raw material. The harvest period will most likely be when *C. finmarchicus* is highly abundant in surface waters. The content of lipids and astaxanthin is highly variable during this period (Paper I and II):

- The content of n-3 fatty acids varies considerably depending on the phytoplankton present during this period. The content of C20:5 n-3 is most dominant during the early bloom of diatoms. The content of C18:4 n-3 and C22:6 n-3 increases later in the spring caused by a bloom of dinoflagellates and other flagellates (*Phaeocystis pouchetii*).
- The lipid content increases from February to June when they feed on phytoplankton blooms, and our results suggest that some CV *C. finmarchicus* migrate to deeper waters when their lipid content reaches a high level in May. CV that do not reach this lipid level will moult to females and start a new generation.
- The content of astaxanthin in *C. finmarchicus* increases from March to June off the coast of Norway and suggests a photo-protective role of astaxanthin caused by the increase in solar irradiation. However, the content of astaxanthin and the fraction of free astaxanthin show high variations from day to day which are not explained by changes in light conditions. These changes are more likely a result of feeding and further synthesis of astaxanthin.
- The abundance of *C. finmarchicus* at the surface can vary greatly between years in the Trondheimsfjord, and could probably be the case in other fjords as well. Harvesting of a small population would not be sustainable.

Autolytic enzymes rapidly degrade proteins and lipids in *C. finmarchicus* post-harvest. The biochemical post-harvest stability of *C. finmarchicus* was studied after denaturation of autolytic enzymes by heat treatment and temporary inactivation of autolytic enzymes by freezing and freeze drying (Paper III and IV).

- The activity of autolytic enzymes is challenging to control by freezing and freeze drying. Storage of untreated biomass at -20°C will result in a decreased content

of phospholipids within 4 months of storage, and freeze drying could also result in a reduced content of phospholipids. Time and temperature are probably the most important factors when trying to control autolytic activity in *C. finmarchicus*.

- Autolytic enzymes must be denatured to maintain phospholipids in the biomass of *C. finmarchicus*, and an instant heat treatment of 70°C for 5 min directly after harvest is sufficient to stop the degradation of phospholipids. The biomass should be heat treated on board the vessel directly after harvest to maintain the content of phospholipids in the harvested biomass.
- Heat treatment (70°C, 15 min) of biomass results in a stable phospholipid content and a low content of free fatty acids for at least 12 months of storage at -20°C. This further illustrates that heating is a excellent tool for preservation for preservation of harvested biomass.
- The content of astaxanthin and n-3 PUFA in both untreated and heat treated biomass is quite stable for at least 12 months of storage at -20°C, indicating an oxidatively stable product.
- The rapid decrease in phospholipids caused by autolytic enzymes is a challenge when sampling biological specimens for scientific studies. The samples have to be frozen immediately to ensure that the content of lipid classes is representative of live specimens. The temperature must also be below -70°C to ensure that the enzyme activity is very low. Such a low temperature could be obtained with liquid nitrogen (-196°C) or with dry ice (-78°C). Without these facilities, it can be difficult to obtain a sufficient low temperature onboard a ship, and one alternative is to conserve samples directly in organic solvents. It is also common to freeze dry samples before analysis. It is important that the temperature of the product during freeze drying sufficiently low. If a low temperature is not achieved, freeze drying should be omitted.

3.2 Future work

In order to exploit a new species, it is important to establish knowledge of the life strategies of the resource and to evaluate the consequences of harvesting. *C. finmarchicus* is an ubiquitous part of the food chain, and it will be important to develop ecological models to predict the effects of harvesting. It is also important to have basic knowledge on the biochemistry of this species. The lipid metabolism of *C. finmarchicus* has been thoroughly reviewed by Sargent and Henderson (1986). However, some questions still remain unanswered. The synthesis of fatty alcohols is thought to occur from protein and carbohydrate precursors *de novo*. The results from Paper I suggest that *C. finmarchicus* use fatty acids from the diet for the synthesis of fatty alcohols. Further research should address the different strategies of lipid metabolism for synthesis of fatty alcohols. It is also unclear if *C. finmarchicus* has the ability to synthesise C22:6 n-3 which is an important part of phospholipids. *C. finmarchicus* can contain a high fraction of C22:6 n-3, but it is not clear where the fatty acid is stored other than in PC, PE and WE. Research should further elucidate the physiological role of C22:6 n-3 in *C. finmarchicus*.

The metabolism of carotenoids in *C. finmarchicus* has not received much attention in the literature, and more studies on the content of astaxanthin in *C. finmarchicus*

from natural waters are therefore needed. The role of the astaxanthin molecule in *C. finmarchicus* is not clear and could be quite diverse. Astaxanthin is mostly present in the esterified form, and the reason for this is also not known. Research should try to elucidate the role of astaxanthin in *C. finmarchicus* under different conditions of light and food.

When it comes to the processing of biomass there are still some unanswered questions. The enzymatic activity should be better studied, including the characteristics of the enzymes and their optimum temperature and pH. The proteases of *C. finmarchicus* have been subjected to research and further work should focus on the characteristics of the lipases and phospholipases of *C. finmarchicus*. This could help both to find characteristics of commercially interesting enzymes and to find the optimum temperature for enzyme inhibition in *C. finmarchicus*.

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Paper I

Dynamics in lipid contents and biomass of *Calanus finmarchicus* (copepodite V) in a Norwegian fjord

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Abstract

Calanus finmarchicus is the dominant zooplankton species in the North Atlantic. This zooplankton is also of interest for commercial harvesting due to its high abundance and biochemical contents. In the present study copepodite stage V of *C. finmarchicus* was sampled at different depths from January to June through 2009, 2010 and 2011 in the Trondheimsfjord (63°29'N 10°18'E). The fatty acid compositions of individual copepods and of the seston were analyzed. It was found that the fatty acid profile of the copepods was well related to the fatty acid profile of its potential food. The sampled *C. finmarchicus* contained 42% n-3 polyunsaturated fatty acids of total fatty acids (March-May). This study indicates that the onset of vertical migration of stage V, which was observed in May, has a strong link to the production of phytoplankton and the lipid accumulation in *C. finmarchicus*. The content of the fatty acids 14:0 and 16:0 in the specimens did not increase from February to May in surface waters. This indicates that these fatty acids in the diet were used as precursors for biosynthesis of the fatty acids 20:1 n-9 and 22:1 n-11, and fatty alcohols.

A possible harvesting season of *C. finmarchicus* could be when the species is abundant in surface waters; the content of n-3 fatty acids will vary trough this season. The peak abundance of *C. finmarchicus* in the spring varied substantially between the years studied.

Key words:

Calanus finmarchicus, lipids, fatty acids, PUFA, wax esters, phytoplankton lipids, lipid metabolism, spring variation, vertical migration

Abbreviations

Chl *a* Chlorophyll *a*

C5 Copepodite stage five

MUFA Mono unsaturated fatty acids

PUFA Poly unsaturated fatty acids

TFA Total fatty acids

WE Wax esters

Introduction

Calanus finmarchicus (Gunnerus, 1765) is the dominant zooplankton species in the Norwegian and Barents Seas [1]. The annual production in the Nordic Seas is roughly estimated to be 74 million tons by wet weight [2]. The copepod has a generation time of 1-2 years in the Norwegian Sea and a dormancy period in deep waters during winter [3, 4]. *C. finmarchicus* hatch as nauplius larvae and progress through six naupliar stages and five copepodite stages before reaching sexual maturity. The females spawn at the time of the spring bloom, and the offspring feed to obtain their large lipid deposits in summer and autumn [5]. The storage lipids of late copepodites and adults are in the form of wax esters (WE), which are fatty acids esterified with fatty alcohols. WE are the most common storage lipids in herbivorous zooplankton feeding on short blooms of phytoplankton followed by long periods with food shortage [6]. The phospholipids mainly consist of phosphatidyl choline and phosphatidyl ethanolamine [7] and contain a high share of the fatty acids 22:6 n-3 and 20:5 n-3 [8, 9].

Due to the abundance and biochemical components, *C. finmarchicus* is a promising new marine raw material and an interesting source for commercial use. World aquaculture has grown steadily in the last 50 years, with an annual growth rate of 8.7 percent (excluding China) since 1970. Meanwhile, for the last 10 – 15 years, most of the world fisheries are fully or overexploited, and the maximum sustainable catch has probably been reached, of which 20-30% are used for animal feed [10]. A further growth in the aquaculture of carnivore fish and crustaceans will require alternative sources of feed ingredients. Vegetable sources can replace part of the fishmeal and oil used today, but the need for n-3 long chain polyunsaturated fatty acids (LC-PUFA) like 22:6 n-3 and 20:5 n-3 will require new marine lipid sources [11, 12]. Harvest of zooplankton could be one alternative to meet the increased demand of n-3 LC-PUFA in the aquaculture industry. Atlantic salmon (*Salmo salar*) is capable to utilize oil from *C. finmarchicus* in its diets [13-16], demonstrating the suitability of *C. finmarchicus* as a new marine ingredient in fish feed. Phospholipids in *C. finmarchicus* have high shares of 22:6 n-3 and 20:5 n-3, and could meet the dietary demands for early marine fish larvae, as they require phospholipids [17] in addition to a high content of 22:6 n-3 and 20:5 n-3 [18]. There has been an increasing awareness of the importance of marine lipids in human nutrition, and beneficial health effects have been documented from consumption of n-3 LC-PUFA which are mainly provided by marine lipids [19]. *C. finmarchicus* could also be an interesting additive in human nutrition. Wax esters from *C. finmarchicus* have a low melting point and long chain, monounsaturated fatty alcohols, which makes it interesting in cosmetics, lubricants, and pharmaceuticals, as discussed by Sargent and Henderson [20].

In the present study we examine the size and fatty acid content of copepodite stage V (C5) of *C. finmarchicus* sampled at different depths at a fixed location in the Trondheimsfjord (63°29'N

10°18'E), from January to June in 2009-2011. The main food source of zooplankton is phytoplankton and protozoa. The stock and condition of *C. finmarchicus* will hence naturally be affected by the changes in phytoplankton composition. In the Trondheimsfjord the first spring bloom consists mainly of diatoms, while later, dinoflagellates and flagellates dominate the bloom [21]. The dominating fatty acids of diatoms are 16:0, 16:1 n-7 and 20:5 n-3 [22] whilst dinoflagellates have variations of 16:0, 18:4 n-3, 18:5 n-3, 20:5 n-3 and 22:6 n-3 [23, 24]. The profile of the fatty acids in wax esters of *C. finmarchicus* will reflect the fatty acid composition of the dietary phytoplankton [25], but the copepods are also dispersed by currents which gives a spatial and depth variation.

In addition to the dietary fatty acids, *C. finmarchicus* are capable of *de novo* synthesis of fatty alcohols from carbohydrates and proteins [26]. Wax esters contains fatty alcohols synthesized *de novo* esterified with fatty acids, and the fatty alcohols contain fairly stable contents of 20:1 n-9 and 22:1 n-11 [27, 28] in addition to 14:0 and 16:0, while their fatty acid composition partly reflects the diet of the copepods. The fatty acids 20:1 n-9 and 22:1 n-11 are also a prominent share of the wax esters [20, 27] and are synthesized *de novo* by *C. finmarchicus* [26]. However, field observations for documenting biosynthesis are scarce and more detailed information from both field and laboratory studies are needed.

The fatty acid composition of *C. finmarchicus* at different depth could also give more information on the vertical migration for dormancy of this copepod in the Trondheimsfjord. This zooplankton needs sufficient lipid storage to overcome dormancy, vertical upwards migration, molting and production of gonads [29]. A threshold lipid level is among several theories suggested as a trigger for vertical migration and dormancy in *C. finmarchicus* [30].

The aim of the present study was to study the lipid variations in *C. finmarchicus* from January to June, before and during the spring phytoplankton blooms, in the Trondheimsfjord. The copepods sampled from different depths were compared on individual level with regard to fatty acid composition and size, and compared to the fatty acid profile of the phytoplankton present.

Material and methods

Sampling procedure

Plankton samples were collected in 2009, 2010 and 2011 (Table 1) at a fixed position in the Trondheimsfjord (63°29'N 10°18'E) between 10:00 h and 14:00 h, from the vessel R/V Gunnerus. The sample location was chosen because it is fairly representative for the water masses in the main body of the fjord [31]. The samples were collected using a multiple opening and closing net system (patent no. NO318542, square opening 0.0625 m^2, 200 µm mesh), mounted on a conductivity temperature depth instrument (CTD, Seabird electronics Inc., USA).

The multinet was hauled vertically and each separate net were opened and closed at selected depth intervals (440 (seabed)-300 m, 300-100 m, 100-50 m and 50-0 m) operated from an on-board control unit. The sampling was repeated three times for the following analyzes.

Samples for determination of species, stage composition and enumeration for an estimate of abundance and stage composition were preserved with buffered formalin (4%) in seawater and stored at room temperature.

Samples for lipid analysis of *C. finmarchicus* were collected as described above, but kept alive in containers with seawater until arrival at the laboratory for further sub sampling (10°C). Viable *C. finmarchicus* from each depth range (5-10 individuals), if available, were then transferred to vials and stored at -80°C under nitrogenous gas (N_2) until analysis.

Samples for biometric measurements from image analysis were collected as described above and kept in containers with seawater (approx. 20L) until arrival at the laboratory for further processing. The containers were placed at 4°C (±2) until sampling for biometry.

Seston samples for lipid analysis (Table 1) of surface water were obtained from seawater pumped from approximately 1.5 meters depth into a reservoir. The water was screened through a plankton net (nominal 200 µm) before feeding to a flow-through centrifuge by gravity at a flow rate of 0.65-0.85 L min^{-1}. The centrifuge was spun at 5500 rpm, and the seston was removed from the centrifugal bowl (D = 10.5 cm) at intervals. Samples for lipid and fatty acid analysis were immediately frozen and stored at -80°C under N_2. Water samples from 0, 3 and 10 meters for analyzes of chlorophyll *a* (Chl *a*) were collected using Niskin water bottles (30 L), screened with a plankton net (nominal 200 µm) and kept in dark containers until filtration. The seston was harvested on Whatman GF/F glass-fiber filters.

Analytical procedure

Live *C. finmarchicus* were classified to developmental stages under a dissecting microscope (Leica MZ6, Leica Microsystems GmbH, Wetzlar, Germany), based on morphological criteria [32] and size [33]. We did not separate *C. finmarchicus* from *C. helgolandicus* when sorting live copepods, but analyzes of the curvature of the basiopod of the 5^{th} pair of swimming legs in *Calanus sp.* from the fixed samples showed very low abundances of *C. helgolandicus* (0-10% compared to *C. finmarchicus*) [34, 35].

For biometric analysis *C. finmarchicus* was placed under a dissecting microscope (Leica MZ125, Leica Microsystems, GmbH, Wetzlar, Germany). Images were captured at fixed

magnifications with a still-video camera (Sony DFW-SX900, Sony Corp, Japan) controlled by the software Fire-i v.3.01.0.111 (Unibrain Inc., San Diego, USA) operated from a computer after anesthetizing the copepods with tricaine methanesulfonate (Finquel™, Argent Laboratories, Redmond, USA; 1.5g/L stock solution in seawater) and orienting them in a fixed position. Biometrical analysis of the copepods were done manually in the software ImageJ 1.43u (National Institutes of Health, Bethesda, USA) with the aid of a graphic tablet (Cintiq 12wx, Wacom Co. Ltd., Saitama, Japan). The volume of oil sac and prosome was calculated according to Miller et al. [36] based on measurement of the length of prosome and oil sac as well as the corresponding area of the two compartments. The software was scaled by measuring an image of a calibration slide (E. Leitz, Wetzlar, Germany) captured at the same magnification as the copepods.

Chlorophyll *a* was extracted in methanol and quantified using a fluorometer (Turner Designs) according to Strickland and Parsons [37].

Total lipids of the seston were extracted and determined gravimetrically according to Bligh and Dyer [38] with modifications described by Jakobsen et al. [39]. Fatty acid methyl esters from extracted lipid were prepared according to Metcalfe et al. [40], and were analyzed in duplicates. A modified direct trans methylation procedure from Abdulkadir and Tsuchiya [41] was used to analyze the fatty acids of individual *C. finmarchicus*. Individual copepods were weighed (UMX2 Ultra-microbalance, Mettler Toledo, Columbus, USA), added 0.5 mL isooctane with C19:0 as an internal standard (Nu-Chek Prep, Japan) followed by 0.2 mL 14% BF_3 in methanol, and heated for two hours (100°C). The tube was cooled on ice, added 0.1 mL isooctane and 0.2 mL distilled water, and mixed with a vortex mixer for 1 min. After centrifugation (1640 G, 3 min) the upper hexane phase was transferred to vials and analyzed with a gas chromatograph (Perkin Elmer AutoSystem XL) run by the software TotalChrom v. 6.3.1 (both Perkin Elmer Inc., Waltham, USA). The instrumental conditions were identical as in the study by Bergvik et al. [42]. Direct methylation for preparing fatty acids of individual *C. finmarchicus* was validated by comparing with the conventional methods of Bligh and Dyer [38] and Metcalfe [40]. Five individuals of *C. finmarchicus* were analyzed with direct methylation and 3 batches with 10 individuals were analyzed with the conventional method. Direct methylation showed slightly higher content of total fatty acids compared to the conventional method, indicating that the method was more efficient in extracting fatty acids from *C. finmarchicus*.

Statistics

Kolmogorov Smirnov was used to test for normality. Some groups of samples were not normally distributed even after log transformation, and the homogeneity of variances were not equal.

Non-parametric analyzes were thereby used for all the statistical work in this paper. Kruskal Wallis was used to compare samples, and Mann Whitney U test was used to compare all samples for significant differences when Kruskal Wallis resulted in a significant difference ($P<0.05$). The significant level for difference in Mann Whitney U test was adjusted with Bonferroni correction and resulted in significant levels of $P<0.008$ for 6 groups and $P<0.013$ for 4 groups. The sample groups varied in size and the lowest and highest number of the groups is specified in each figure. All statistical analyses were performed using of SPSS Statistics v. 17.0 (SPSS Inc., Chicago, USA). Samples are presented as average of the mean and error bars as standard errors of the mean (SEM). All Figures are made in SigmaPlot v. 10.0 (Systat Software Inc., San Jose, USA).

Results

C. finmarchicus was sampled from January to June during three years (2009 – 2011) in the Trondheimsfjord at four different depth intervals. The concentration of chlorophyll *a* (Chl *a*) was also measured through the sampling period to follow the phytoplankton biomass fluctuations. The females spawned early in the spring and the smaller stages of *C. finmarchicus* (copepodite stage 1 – 3) were registered in April all years of the study. The appearance of the smaller stages coincided with the maximum concentration of Chl *a*, although the Chl *a* concentration was low in 2010 compared to the 2 other years (Fig. 1A). The highest abundances of *C. finmarchicus* (sum of all stages) were observed during April and May in 2009 and 2011 (Fig. 1B), whereas there was a minimal number of *C. finmarchicus* in 2010, similar as for the concentration of Chl *a*. The maximum numbers of *C. finmarchicus* in 2009 were 4 times higher compared to 2011. The concentration of chlorophyll *a* (Chl *a*) in the upper 10 m (Fig. 1A) was positively correlated with the abundance of *C. finmarchicus* in the upper 50 m, the phytoplankton bloom measured as Chl *a* were followed by an increase in numbers of *C. finmarchicus*. The abundances of copepodite stage 4 (C4), copepodite stage 5 (C5), females and males (Fig. 2) in the surface (0-50 m) show that C4 and C5 peaked in late April and May all years. Following the peak of C5 was a smaller peak of females, simultaneous as the numbers of C5 were increasing in deeper waters (50 – 440 m). The numbers of males was low and showed little variation, but a small increase was seen in February and March from 300 m and upwards to the surface. The females also showed a small increase in February and March. In January and February of all years, C5 resided mainly in intermediate and deeper layers, and was present in low numbers during April and May, but increased again from May to June when some of the C5 started the migration to deeper layers. C5 was the dominant stage in the deepest layer. The variations between years in the numbers of C5 at intermediate depths and close to the seabed were not as pronounced, compared to that at the surface.

The total lipid content (Fig. 3A) and total fatty acid (TFA) content (Fig. 3B) of the seston showed pronounced variations both through the year and between years. The lipid content was highest in April 2009, in May 2010 and in June 2011 and ranged from 2-12% of dw. TFA were on average 29% of the total lipid.

The average content of total fatty acids (TFA, mg g^{-1} dw) in C5 from both 0-50 m (Fig. 4A) and 300-440 m (Fig. 4B) did not change much from January to June in 2009-2011. The dry weight per individual C5 in the upper 50 m increased steadily from February to June. The dry weight in January was higher compared to February, but not statistically different from that in April to June (Fig. 2C). The dry weight of C5 close to the seabed (300-440 m) was also increasing from January to May, but showed the same dry weight in June (Fig. 2D). The content of total fatty acids (TFA) per individual in surface and close to seabed showed a similar pattern of variation as the dry weight (Fig. 2E-F), and a correlation was found between individual dry weight and TFA contents (r_s=0.87, N=389, P< 0.001) (Fig. 4G). The body volume of C5 also correlated with the oil sac volume (r_s=0.80, N=393, P<0.001) (Fig. 4H). The dry weights of C5 were significantly smaller in the surface (0-50 m) compared to both at intermediate depths and close to the seabed (Fig. 3A). The same trend was apparent for the mean body volume (Fig. 3B) and the individual content of TFA and the oil sac volume, which both increased from surface towards deeper water layers (Fig. 3C-D).

The fatty acid composition of the seston showed a similar pattern of variation for all years, although with some variation between years (Fig. 4A). The dominant fatty acids of the seston was 22:6 n-3, 20:5 n-3, 18:4 n-3, 16:1 n-7, 18:1 n-9, 18:0, 16:0 and 14:0. The fraction of the flagellate fatty acid, 18:4 n-3 showed the highest variation between years, with a share of 18:4 n-3 of 5% in 2010 and 25% in 2009. The contents of the diatom fatty acid 16:1 n-7 showed the same pattern during spring in all three years, with a higher content in the middle of April and in the start of June.

The content of the n-3 polyunsaturated fatty acids (PUFA) 22:6 n-3, 20:5 n-3 and 18:4 n-3 in the surface C5 individuals decreased from January to February, but increased from February to March and remained relatively stable until June (Fig. 4B). The content of 16:1 n-7, 18:1 n-9, 14:0 and 16:0 decreased from January to February and an increase again towards June. The minor fatty acids constituting on average under 1% of TFA are not presented. The content of the different fatty acids in C5 close to seabed (Fig. 4C) showed a general increase of the fatty acids originating from the diet. The fatty acids 16:1 n-7 and 18:4 n-3 are fatty acids from diatoms and flagellates, respectively. Most evident was the increase of 18:4 n-3 in April, May and June, reaching 13% of TFA from zero, suggesting that surface C5 that have grazed on the spring bloom of phytoplankton had migrated towards the seabed. A raise was also seen in the content

of 22:6 n-3, 20:5 n-3 and 16:1 n-7 in May. The contents of 18:1 n-9, 14:0 and 16:0 showed no significant differences from January to June.

The contents of 20:1 n-9 and 22:1 n-11 in C5 in the surface were declining from January to February (Fig. 6A and C). During February to June there were no changes in the content of these fatty acids at 0-50 m depth but we found a higher level of 20:1 n-9 and 22:1 n-11 close to seabed (Fig. 6B and D). There was a significant increase of 20:1 n-9 and 22:1 n-11 from the surface to deeper waters (Fig. 7E).

The fraction of n-3 PUFA of TFA (Fig. 8A) in C5 *C. finmarchicus* was declining from January to February followed by an increase of 61% until March. The percent share remained unchanged until a decline took place in June. The decline from January to February was caused by a reduced content of 18:4 n-3 while the contents of the fatty acids 20:5 n-3 and 22:6 n-3 did not change. The fatty acid 20:5 n-3 was the cause of the high increase in n-3 PUFA from February to March, while 22:6 n-3 were gradually increasing until May. The share of mono-unsaturated fatty acids (MUFA) (Fig. 7B) in C5 decreased slightly from January to April where after it remained unchanged. The percent content of 20:1 n-9 and 16:1 n-7 did not change much over the period, but the share of 22:1 n-11 shared a similar pattern of variation with time as MUFA.

The dry weights of C5 in each depth at the separate months are shown in Fig. 9A. Similar for all months except for March was that the thinnest C5 was found in the surface, although the biggest were at intermediate depths in January and June. In Fig. 8B the dynamics of 18:4 n-3, 20:5 n-3 and 22:6 n-3 are shown. In January the content of 18:4 n-3 was low through all depths, while the content of 20:5 n-3 and 22:6 n-3 was higher and stable. In February the content of these fatty acids decreased in all depths, which also were seen with dry weight and lipid (Fig. 9). In March the same fatty acids increased in the surface as the fatty acids became available in the seston. Already in April there was an increase of 18:4 n-3, 20:5 n-3 and 22:6 n-3 in intermediate layers that could indicate a vertical migration of some C5 from the surface. In May these fatty acids also increased at the deepest layer. The content of the fatty acids 20:1 n-9 and 22:1 n-11 in C5 (Fig. 8C) were lower in the surface through all months. The content in the surface decreased from January to February, but remained high in deeper layers. In March the content of 20:1 n-9 and 22:1 n-11 decreased also in deeper layers. In April and May the content of these fatty acids were increasing in the deeper layers, but remained low in the surface.

Discussion

The contents of lipid and fatty acids in C5 of *C. finmarchicus* in the present work reviled an interaction between the phytoplankton and *C. finmarchicus,* and that the lipid and fatty acid accumulation of C5 could be the trigger for vertical migration in the spring. Several theories on

the mechanism that triggers the onset of dormancy in *C. finmarchicus* have been suggested, where food availability, temperature, photoperiod and lipid accumulation are among the suggested cues for entering dormancy [30, 43, 44]. In the present study there was an increased abundance of C5 in deep waters (300-440 m) in May indicating the start of vertical migration. We found that the individual size of C5 in surface waters was smaller than the individuals from deep waters and is found in previous studies [5, 45]. The total fatty acid contents and volume of the oil sac of C5 also increased from the surface waters towards deeper waters. This has been observed previously for both C5 [29, 46] and females [47], and Hirche [45] found larger C5 in deep waters compared to those residing in surface waters. *C. finmarchicus* is dependent on a large lipid reserve to survive dormancy, undertake upwards migration, and in addition to have enough energy for molting and gonad formation [29]. The size and lipid content at each depth in May increased both in the deepest layer as compared to the values in April. This suggests that C5 that migrated towards the depth have higher lipid contents and are bigger compared to C5 remaining in surface waters. The lipid level could thus be the trigger for vertical migration and the onset of dormancy as discussed by Irigoien [30].

The fatty acid composition of C5 stages of *C. finmarchicus* can also contribute to a better understanding of the onset of dormancy and the relationship between *C. finmarchicus* and the phytoplankton present. The fatty acids of the seston clearly showed the signature fatty acids of diatoms and flagellates over the season (16:1 n-7 and 18:4 n-3 respectively), revealing a bloom of diatoms at the end of March and a bloom of flagellates at the start of June. Sakshaug and Myklestad [21] found a bloom of diatoms in the Trondheimsfjord at the end of March both in 1970 and 1971. Spring blooms of dinoflagellates showed high variations between the two years of their study, but dinoflagellate blooms followed the diatom blooms both years. *C. finmarchicus* feeds mainly on phytoplankton and the signature fatty acids of the phytoplankton are directly incorporated in their wax esters [25, 48]. We found that 22:6 n-3, 20:5 n-3, 18:4 n-3 and 16:1 n-7 were the dominating fatty acids from the phytoplankton that got incorporated in the C5 of *C. finmarchicus*. The increase of these fatty acids in *C. finmarchicus* was thereby related to the available fatty acids in the seston. The increase of the main n-3 PUFA, 18:4 n-3, 20:5 n-3 and 22:6 n-3, in the deep waters close to seabed was pronounced in May, suggesting that the deep water C5 had recently fed on phytoplankton in the surface. Most prominent was the content of 18:4 n-3 that was nearly at zero level in January to March close to seabed, reaching 15% of TFA during May. The content of 18:4 n-3 and 20:5 n-3 was in fact higher in C5 from deep waters compared to the surface waters. This might suggest that when C5 copepods in the surface reach a certain content of wax esters after accumulation of phytoplankton, they will migrate towards deeper waters. The onset of migration was about one month after the phytoplankton bloom, suggesting a strong link between the production of phytoplankton, lipid accumulation and onset of migration.

Although some C5 descended to deeper waters in May, there was concurrently an increase of the numbers of females in the surface. This could indicate that while some C5 starts a vertical migration, others molt to females. We suggest that the lipid content will decide whether a C5 will migrate or molt. If the lipid content is above a certain level, the C5 will descend to deeper waters. If the lipid level has not been reached the C5 will molt to females which will start a new generation of *C. finmarchicus*. Miller et al. [36] found that large C5 in the surface waters in December molted to females while the smaller C5 remained in surface waters waiting for food until March, when they again found larger C5 which they attribute to be the new generation C5. The results from our study suggest that lipid accumulation has a strong connection with the onset of dormancy. Johnson et al. [49] did not find an environmental factor that explained the onset of dormancy in *C. finmarchicus*, and suggested that lipid accumulation could be an explanation.

In our study most of the fjord population of *C. finmarchicus* had ascended to the surface by March. C5 stages of *C. finmarchicus* that have ascended from deeper water will probably use their lipid reserves to molt into adults and development of gonads. Gatten et al. [50] showed that one half of the wax ester reserves in *C. helgolandicus* were utilized when C5 matured to females and formed gonads. Jonasdottir [29] also suggested that the main part of the lipid reserve was used for vertical migration and gonad formation in *C. finmarchicus*. The lipid content and the size of C5 in our study decreased from January to February, but this could also be the result of a lack of food in the surface. Copepods in surface waters has been shown to be higher compared to that of copepods in deeper waters [5] because the metabolism during dormancy at deeper water is low [45]. Hence, *C. finmarchicus* would have to catabolize wax esters to meet the increased energy demand in the surface [51]. Other authors have also found a decrease in size and lipid, but were from December to January [29, 52, 53] and from February to May [46]. Irigoien et al. [54] also found a decrease in carbon content in *C. finmarchicus* before the spring bloom appeared. Our data shows a small increase of females in the surface and in intermediate waters and an increase of males in intermediate waters, all in February. We suggest that lipid rich C5 ascends to the surface waters and molts into females while the remaining C5, that has lower lipid contents, must wait for the phytoplankton production in order to molt. The deviations in point of time of depletion of lipid in the studies mentioned above could be caused by different locations of study, which could result in a shift in the point of time for upwards migration. An earlier upwards migration would lead to an earlier depletion of lipids. Females have shown to produce eggs prior to the phytoplankton bloom [55], suggesting that the lipid reserves are used for the production of eggs. However, Koski et al. [56] have shown that the fatty acid profile of eggs from *C. finmarchicus* resembled the fatty acid profile of the seston. The smaller copepodite stages (CI-CIII) were observed during April in all three years in our

study, coincident with the phytoplankton bloom, suggesting that the egg production was taking place before the phytoplankton bloom. The new generation of C5 may be present already in late April, given that sufficient amounts of food are present.

The content of the fatty acids 20:1 n-9 and 22:1 n-11 in the surface living individual C5 was low, but the content was significantly higher close to seabed. When *C. finmarchicus* accumulates energy reserves as wax esters during spring, the formation of fatty alcohols is a necessary step of that synthesis. The proposed route of biosynthesis for fatty alcohols is synthesis of fatty acids and further conversion of the fatty acids into fatty alcohols by a fatty acyl coenzyme A oxido-reductase [20, 57]. The production of wax esters by the copepods is in fact suggested to be limited by this synthesis of fatty alcohols from fatty acids [57, 58]. *C. finmarchicus* contains high amounts of the fatty alcohols 20:1 n-9 and 22:1 n-11 [27, 28, 59]. The fatty acids 20:1 n-9 and 22:1 n-11 are synthesized first with a following reduction to the corresponding fatty alcohols during lipid accumulation. The content of these fatty acids were scarce during the phase of lipid accumulation in surface waters in the present study, and we can assume that all of the synthesized 20:1 n-9 and 22:1 n-11 was converted to the fatty alcohols 20:1 n-9 and 22:1 n-11. When *C. finmarchicus* is descending to deeper waters, the copepods have accumulated enough wax esters to enter dormancy and to hibernate through winter. They have no longer the need for biosynthesis of fatty alcohols and they may instead accumulate the fatty acids 20:1 n-9 and 22:1 n-11 in their WE. The present study shows that the content of the long chain MUFA is accumulating in the copepods at 50-100 m and further in depth.

The fatty acids 20:1 n-9 and 22:1 n-11 is suggested to be synthesized *de novo* from carbohydrate- and protein precursors [6] with the intermediates 14:0, 16:0 and 18:0, that are further elongated and desaturated to form 20:1 n-9 and 22:1 n-11. Also, Henderson and Sargent [60] have shown that the carnivorous copepod *Eucheta norvegica* uses glucose and amino acids in *de novo* biosynthesis of fatty alcohols. In the present study, 14:0, 16:0 and 18:0 in the phytoplankton were present in high contents, e.g. the content of 18:0 was 20% of TFA in March and April and the content of 14:0 and 16:0 was on average 30-50% of TFA during the sampling period. The content of these fatty acids in the copepods did, however, not increase from February to May and the content of 18:0 was scarce. At the same time, the contents of 18:4 n-3, 20:5 n-3 and 22:6 n-3 in the copepods increased, suggesting that the phytoplankton was ingested. The saturated fatty acids 14:0 and 16:0 could then either be catabolized for energy or they could be used as precursors for fatty alcohol synthesis of the longer mono-unsaturated fatty alcohols, 14:0 and 16:0. Some evidence of incorporation of dietary 14:0 into wax esters has been provided, also as fatty alcohol in *C. helgolandicus* [58], and it is also suggested by Kattner and Krause [27]. Sargent and Falk-Petersen [51] suggested the possibility of a further elongation of dietary 14:0 and 16:0, but no evidence was found to support this.

Graeve et al [61] showed that *C. finmarchicus* retained PUFA from a ^{13}C labeled diatom diet, and converted 16:1 n-7 from the diet to the corresponding fatty alcohol. This suggested that *C. finmarchicus* is selectively accumulating and retaining fatty acids in accordance to its metabolic requirements [61]. Sargent and Falk-Petersen [51] also suggested the possibility that synthesis of wax ester is a flexible system in such a way that zooplankton can switch the *de novo* synthesis in relation to a varying input of diet.

The individual content of 20:5 n-3 and 22:6 n-3 decreased from January to February, while the content of the same fatty acids per dry weight was unchanged. From February to March there was a significant increase in 20:5 n-3 and a tendency of increase in 22:6 n-3. The phytoplankton in the surface water contained high fractions of 20:5 n-3 and low fractions of 22:6 n-3, in agreement with the different increase of these fatty acids in the copepods. Phospholipids in *C. finmarchicus* consist mainly of 20:5 n-3 and 22:6 n-3 in the proportions 1:2 [8, 62]. 22:6 n-3 is an essential fatty acid that is important for maintaining specific cellular functions, and Scott et al [63] discussed 22:6 n-3 as an important factor in mobility and migration of *C. finmarchicus*. The fatty acid has also shown to be important in the reproductive capability of copepods [64]. Marine copepods have shown low capabilities for synthesis of 22:6 n-3 and 20:5 n-3, but the need for n-3 PUFA in their phospholipids and for reproduction are vital. Bell et al. [65] showed that female of *C. finmarchicus* had a low ability to convert 18:3 n-3 to 20:5 n-3 and 22:6 n-3. But it does not rule out the possibility that *C. finmarchicus* can synthesize 22:6 n-3 if the need is not met through the diet. Koski et al [56] showed that the fatty acid composition in *Calanus* eggs reflected the diet of the females, and furthermore suggested that hatching success was limited by lipid or PUFA content in the eggs. This further supports that these specimens did not have the ability to efficiently synthetize n-3 PUFA. Nutrition studies on *C. finmarchicus* should be addressed in order to get a better understanding of the lipid metabolism and the importance of 20:5 n3 and 22:6 n3 during ontogenic development.

We suggest that *C. finmarchicus* could be a new future source of marine raw material. *C. finmarchicus* is abundant in surface waters in the spring and summer when the phytoplankton or other potential zooplankton food is present, and can be found in very high concentrations. This short time-window is the most likely period for commercial harvest. The content of n-3 fatty acids in *C. finmarchicus* will vary considerably depending on the phytoplankton present during this period. The content of 20:5 n-3 will be most dominant during the bloom of diatoms, and the content of 18:4 n-3 and 22:6 n-3 will increase later in spring caused by a bloom of diatoms and other flagellates (e.g. *Phaeocystis pouchetii*). The content of WE will also increase during spring, indirectly shown as an increase of total fatty acids in the present work. We suggest that C5 will start the vertical migration when a significant lipid level has been reached and that C5 with lower lipid levels after the phytoplankton bloom will molt to females in the surface and start

a new generation. The vertical migration of C5 is in May in the Trondheimsfjord and a possible harvest should exploit *C. finmarchicus* in the period when it is most abundant and before the majority of the copepods descend to depth. However, the concentration of *C. finmarchicus* in the surface can vary greatly between years in the Trondheimsfjord, which can probably be the case also in other fjords. When this copepod is present in low numbers in a fjord, harvesting can have some impact on the predators of the zooplankton, and harvesting on a low population would maybe not be sustainable. However when *C. finmarchicus* is abundant, harvesting could even be a positive input for the population, leaving more food for the remaining stock. Our results suggest that fjords are not a suited harvesting arena for *C. finmarchicus*, at least not when the abundance is low.

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Fig. 1: Concentration of Chl a (µg L^{-1}) in the upper 10 m from January to June in 2009-2011 (A). Abundance of *C. finmarchicus* (sum of copepodite stage 4 (C4), copepodite stage 5 (C5), females and males) (ind L^{-1}) in 0-50 m (B) from January to June in 2009-2011.

Fig. 2: Abundance of copepodite stage 4 (C4), copepodite stage 5 (C5), females (F) and males (M) (ind L^{-1}) at 0-50 m, 50-100 m, 100-300 m and 300-440 m from January to June in 2009-2011.

Fig. 3: Content of total lipid (mg g^{-1} dw) (A) and total fatty acids (TFA) (mg g^{-1} dw) (B) in seston from March to June in 2009-2011.

Fig. 4: Average total fatty acid (µg g^{-1} dw) of *C. finmarchicus* C5 in 0-50 m (A) and 300-440 m (B) from January to June in 2009-2011. Average dry weight (µg ind^{-1}) of *C. finmarchicus* C5 in 0-50 m (C) and 300-440 m (D) from January to June in 2009-2011. Average total fatty acid (µg ind^{-1}) of *C. finmarchicus* C5 in 0-50 m (E) and 300-440 m (F) from January to June in 2009-2011. Correlation (r_s=0.91, p<0.001, N=389) between dry weight (µg ind^{-1}) and TFA (µg ind^{-1}) in all *C. finmarchicus* C5 in this study (G). Correlation (r_s=0.80, p<0.001, N=393) between prosome volume (mm^3) and oil sac volume (mm^3) in all *C. finmarchicus* C5 with biometric measurements (H). Lower letters indicate significant differences and star indicates no significant difference. The sample sizes in A, C and E ranged from 10-36 individuals, and in B, D and F the sample sizes ranged from 5-20 individuals.

Fig. 5: Average dry weight (µg ind^{-1}) (A), prosome volume (mm^3) (B), TFA (µg ind^{-1}) (C) and oil sac volume (mm^3) (D) in *C. finmarchicus* C5 in 0-50 m, 50-100 m, 100-300 m and 300-440 m depth. Lower letters indicate significant differences and star indicates no significant difference. N equals number of replicates.

Fig. 6: Share of fatty acids (% of TFA) in seston (A) from March to June in 2009-2011. Average content of fatty acids (µg ind^{-1}) in *C. finmarchicus* C5 from January to June through 0-50 m (B) and 300-440 m depth (C). In addition to 20:1 n-9 and 22:1 n-11 the share of these fatty acids is on average over 1% of TFA in *C. finmarchicus*. Lower letters indicate significant differences and star indicates no significant difference. The sample sizes in B ranged from 10-28 individuals and in C the sample sizes ranged from 5-19 individuals.

Fig. 7: Average content of the fatty acid 22:1 n-11 in *C. finmarchicus* C5 from January to June through 0-50 m (A) and 300-440 m depth (B). Average content of the fatty acid 20:1 n-9 in *C. finmarchicus* C5 from January to June through 0-50 m (C) and 300-440 m depth (D). Average content of 20:1 n-9 and 22:1 n-11 in C5 through all depths: 0-50 m, 50-100 m, 100-300 m and

300-440 m. Lower letters indicate significant differences and star indicates no significant difference. The sample sizes in A and C ranged from 10-28 individuals, in B and D the sample sizes ranged from 5-19 individuals and in E the sample sizes ranged from 66-123 individuals.

Fig. 8: Fraction of n-3 polyunsaturated fatty acids (PUFA) (A) and monounsaturated fatty acids (MUFA) (% of TFA) (B) in *C. finmarchicus* C5 in 0-50 m from January to June 2009-2011. The main n-3-PUFA and MUFA are also included. Lower letters indicate significant differences and star indicates no significant difference. The sample sizes in A and B ranged from 10-28 individuals.

Fig. 9: Dry weight (mg ind^{-1}), total fatty acids (TFA, µg ind^{-1}) (A), content of 18:4 n-3, 20:5 n-3 and 22:6 n-3 (µg ind^{-1}) (B) and content of 20:1 n-9 and 22:1 n-11 (µg ind^{-1}) (C) in C5 of *C. finmarchicus* at 0-50 m, 50-100 m, 100-300 m and 300-440 m from January to June. There are two x-axes in Fig. 9A where dry weight relates to the lower x-axis and TFA relates to the upper x-axis. All numbers are the average of 2009-2011. The sample sizes ranged from 10-15 in January, 13-25 in February, 12-26 in March, 5-27 in April, 19-36 in May and 5-10 in June.

Table 1: Overview over sampling dates of copepods for lipid analyzes and biometric measurements and seston for lipid analyzes in 2009-2011.

Year	Month	Seston samples for lipid analyzes	Copepod samples for lipid analyzes	Copepod samples for biometric measurements
2009	Feb		23.02.09	
	March	13.03.09	30.03.09	
		17.03.09		
		24.03.09		
	April	01.04.09	27.04.09	
		22.04.09		
		28.04.09		
	May	19.05.09	18.05.09	
		25.05.09		
	June	03.06.09		
2010	Jan		26.01.10	
	Feb	28.02.10	18.02.10	18.02.10
	Mar	26.03.10	02.03.10	02.03.10
			15.03.10	
	Apr	07.04.10	06.04.10	
		28.04.10*	28.04.10	
	May	18.05.10*	18.05.10	18.05.10
	June	09.06.10		
2011	Jan		25.01.11	25.01.11
	Feb		14.02.11	
	Mar	09.03.11	07.03.11	
		15.03.11	22.03.11	
		29.03.11		
	Apr	07.04.11	26.04.11	
	May	05.05.11		
		09.05.11*	09.05.11	
		30.05.11*	30.05.11	
	June	20.06.11*	20.06.11	20.06.11

Figure 1

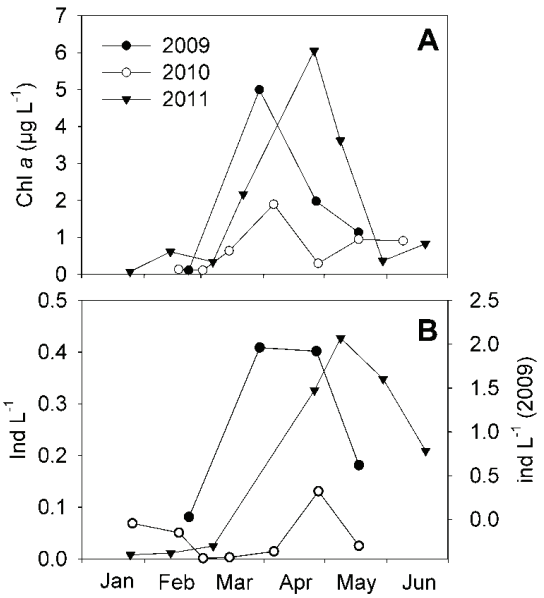

A
2009
2010
2011
Chl a (µg L-1)
B
Ind L-1
ind L-1 (2009)
Jan
Feb
Mar
Apr
May
Jun

Figure 2

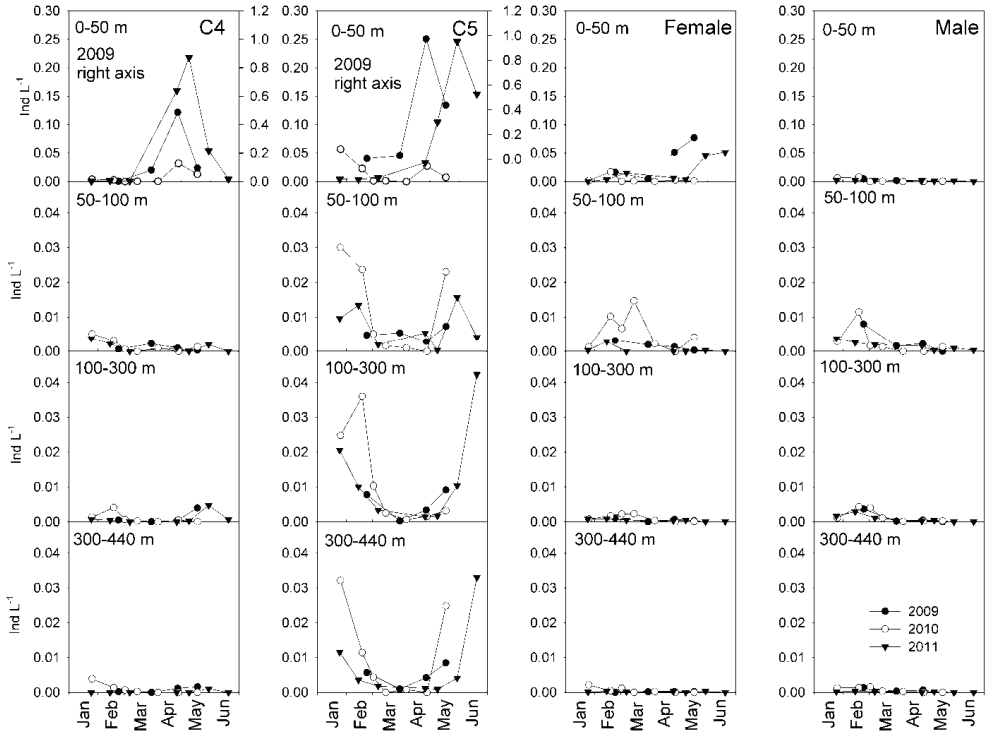

0-50 m
C4
2009
right axis
50-100 m
100-300 m
300-440 m
Ind L⁻¹
0-50 m
C5
2009
right axis
50-100 m
100-300 m
300-440 m
0-50 m
Female
50-100 m
100-300 m
300-440 m
0-50 m
Male
50-100 m
100-300 m
300-440 m
2009
2010
2011
Jan
Feb
Mar
Apr
May
Jun

Figure 3

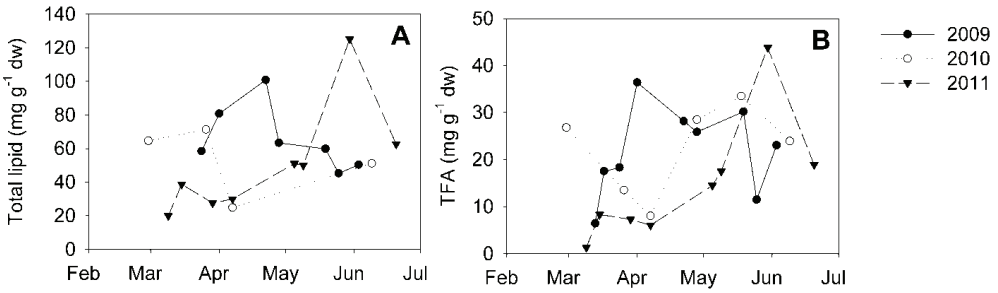

A
B
Total lipid (mg g-1 dw)
TFA (mg g-1 dw)
0
20
40
60
80
100
120
140
10
30
50
Feb
Mar
Apr
May
Jun
Jul
2009
2010
2011

Figure 4

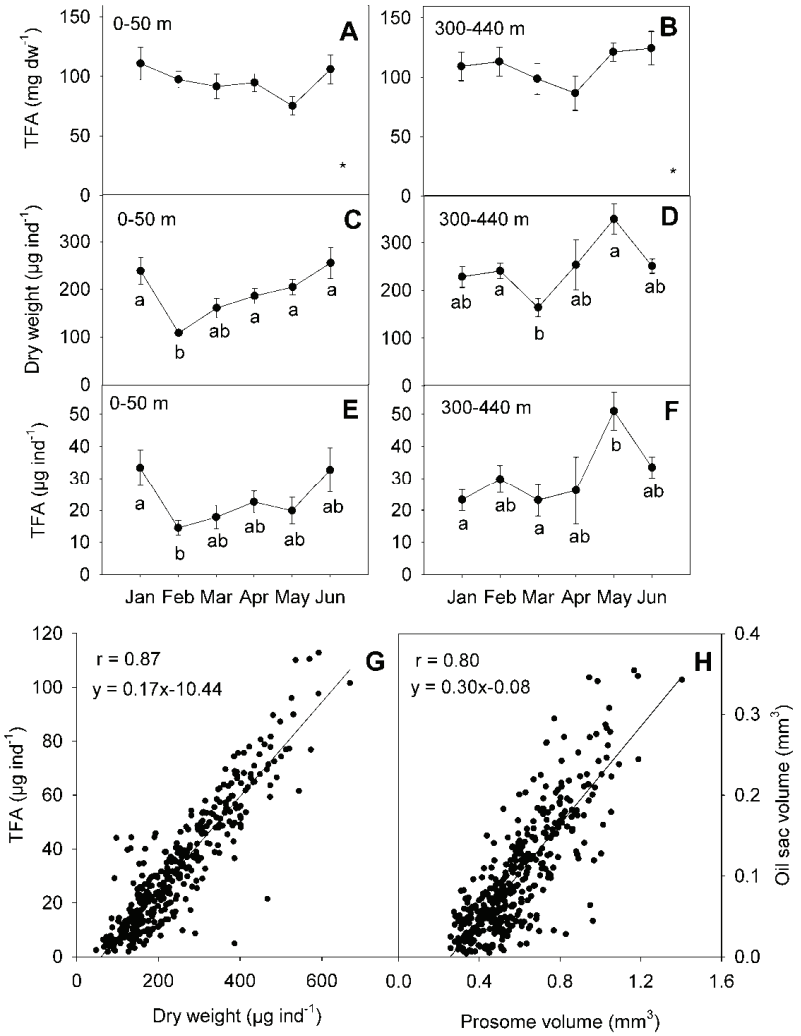

0-50 m
A
300-440 m
B
TFA (mg dw-1)
0-50 m
C
300-440 m
D
Dry weight (µg ind-1)
0-50 m
E
300-440 m
F
TFA (µg ind-1)
Jan Feb Mar Apr May Jun
Jan Feb Mar Apr May Jun
r = 0.87
y = 0.17x-10.44
G
r = 0.80
y = 0.30x-0.08
H
TFA (µg ind-1)
Oil sac volume (mm3)
Dry weight (µg ind-1)
Prosome volume (mm3)

Figure 5

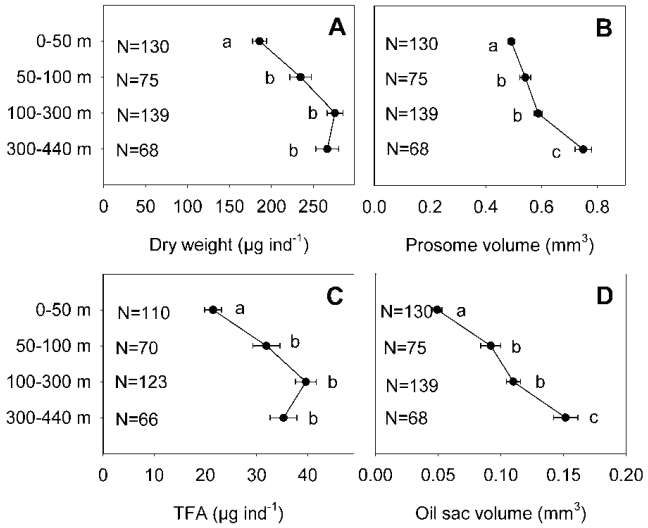

A
B
C
D
0-50 m
50-100 m
100-300 m
300-440 m
N=130
N=75
N=139
N=68
N=110
N=70
N=123
N=66
a
b
b
b
a
b
b
c
a
b
b
b
a
b
b
c
0 50 100 150 200 250
Dry weight (µg ind-1)
0.0 0.2 0.4 0.6 0.8
Prosome volume (mm3)
0 10 20 30 40
TFA (µg ind-1)
0.00 0.05 0.10 0.15 0.20
Oil sac volume (mm3)

Figure 6

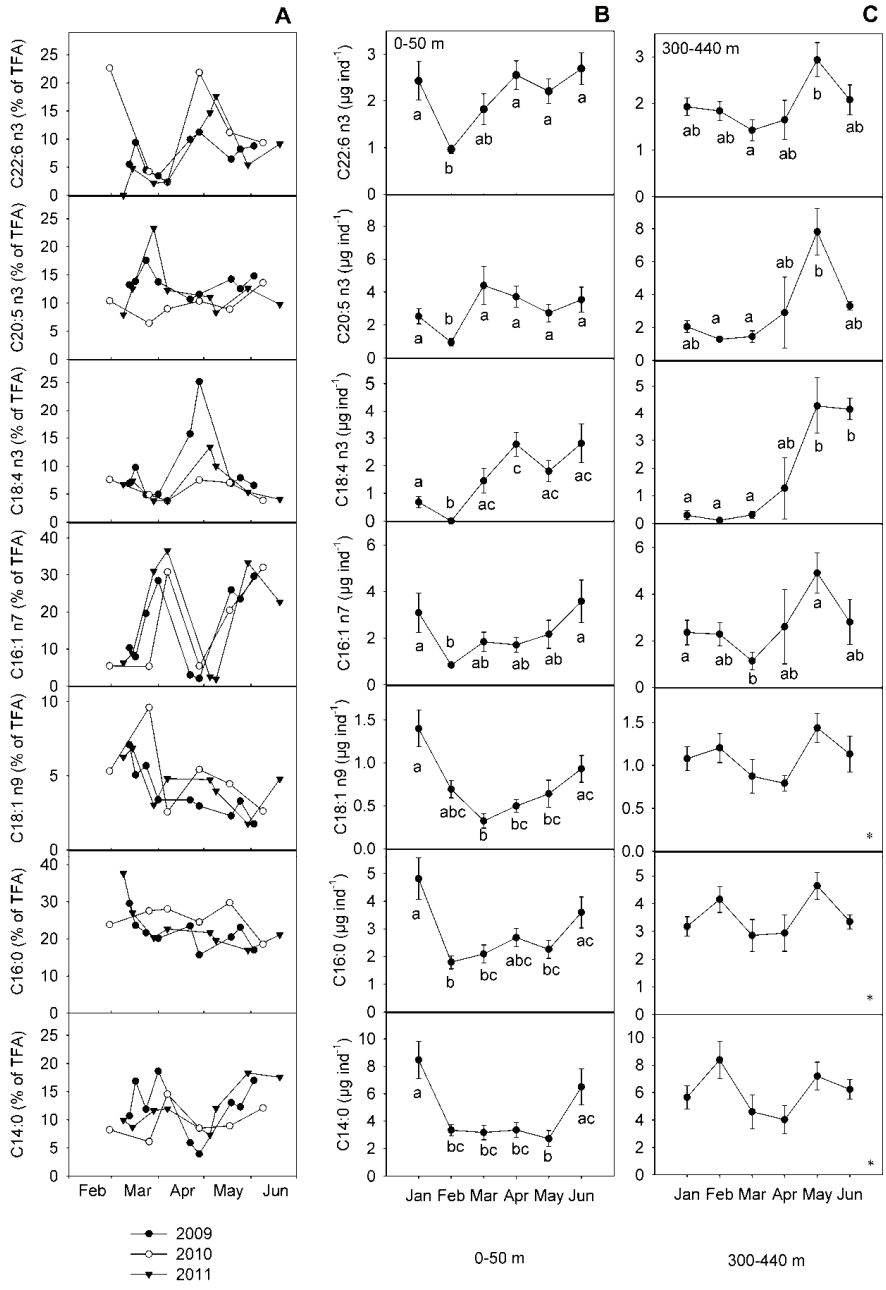

A
B
C
0-50 m
300-440 m
C22:6 n3 (% of TFA)
C20:5 n3 (% of TFA)
C18:4 n3 (% of TFA)
C16:1 n7 (% of TFA)
C18:1 n9 (% of TFA)
C16:0 (% of TFA)
C14:0 (% of TFA)
C22:6 n3 (µg ind-1)
C20:5 n3 (µg ind-1)
C18:4 n3 (µg ind-1)
C16:1 n7 (µg ind-1)
C18:1 n9 (µg ind-1)
C16:0 (µg ind-1)
C14:0 (µg ind-1)
Feb Mar Apr May Jun
Jan Feb Mar Apr May Jun
2009
2010
2011
0-50 m
300-440 m

Figure 7

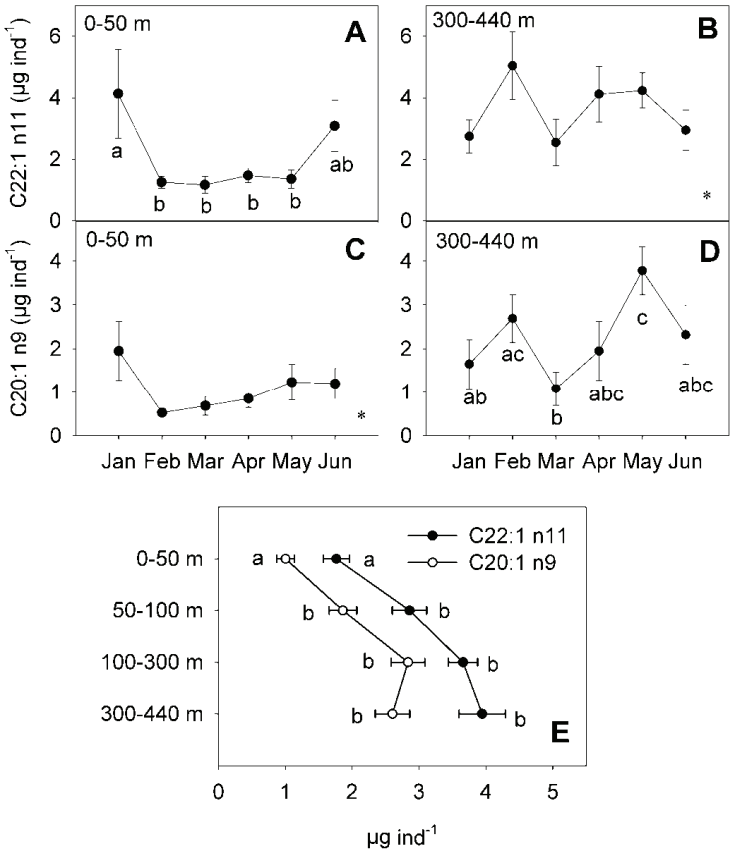

0-50 m
A
300-440 m
B
0-50 m
C
300-440 m
D
C22:1 n11 (µg ind-1)
C20:1 n9 (µg ind-1)
Jan Feb Mar Apr May Jun
Jan Feb Mar Apr May Jun
C22:1 n11
C20:1 n9
0-50 m
50-100 m
100-300 m
300-440 m
E
µg ind-1

Figure 8

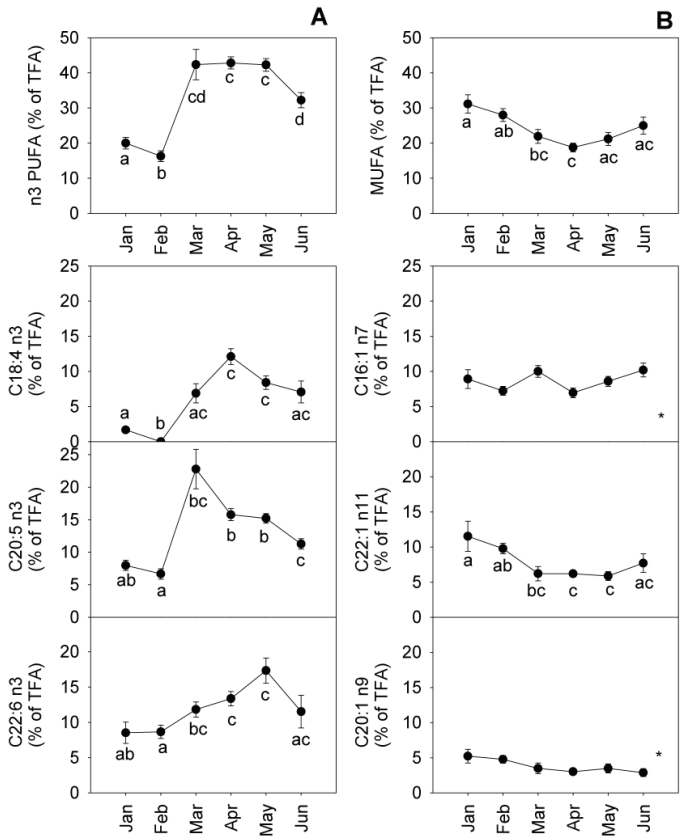
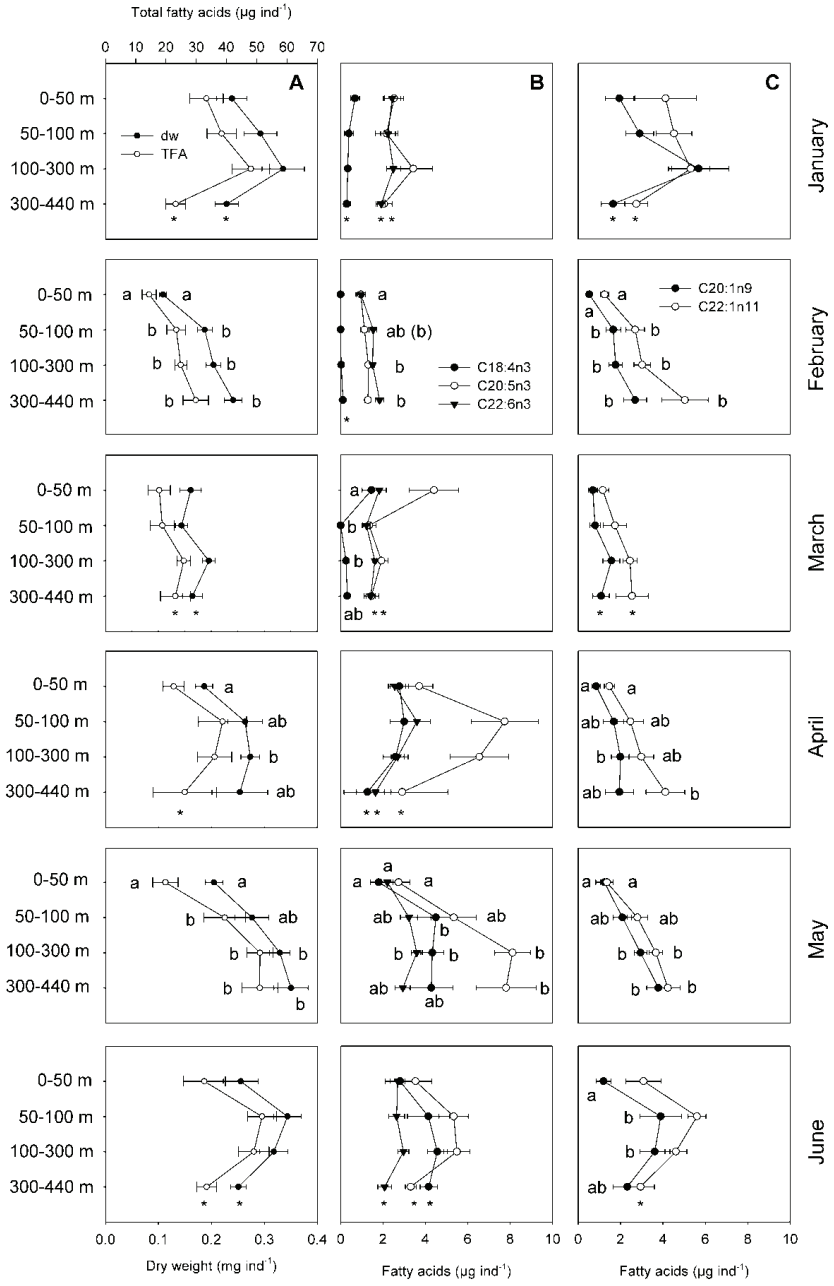

A
B
n3 PUFA (% of TFA)
MUFA (% of TFA)
C18:4 n3 (% of TFA)
C16:1 n7 (% of TFA)
C20:5 n3 (% of TFA)
C22:1 n11 (% of TFA)
C22:6 n3 (% of TFA)
C20:1 n9 (% of TFA)
Jan
Feb
Mar
Apr
May
Jun

Figure 9


Total fatty acids (µg ind-1)
A
B
C
0-50 m
50-100 m
100-300 m
300-440 m
dw
TFA
C18:4n3
C20:5n3
C22:6n3
C20:1n9
C22:1n11
January
February
March
April
May
June
Dry weight (mg ind-1)
Fatty acids (µg ind-1)
Fatty acids (µg ind-1)

Paper II

Is not included due to copyright

Paper III

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Properties of *Calanus finmarchicus* biomass during frozen storage after heat inactivation of autolytic enzymes

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ABSTRACT

Calanus finmarchicus is a marine zooplankton of interest for the aquaculture industry, as well as for nutraceuticals and the cosmetic industry. The chemical composition of *C. finmarchicus* rapidly changes *postmortem* due to autolytic processes; in particular phospholipids rapidly degrade to give free fatty acids. The aim of this study was to inactivate autolytic enzymes in *C. finmarchicus* by applying heat (72 °C, 5–30 min) through mixing with boiling, fresh water, and further to explore the effects of heat (70 °C, 15 min) combined with long time storage (−20 °C, 12 months) of treated and untreated material. Heat treatment (5 min) inactivated all tested enzymes and maintained the initial amount of phospholipids, total lipids and crude protein. Storage of untreated material led to complete degradation of all phospholipids, whereas heat treatment resulted in a stable product containing the initial amount of phospholipids and astaxanthin.

1. Introduction

Calanus finmarchicus is the dominant zooplankton species in the Norwegian and Barents Seas (Planque & Batten, 2000). The annual production in the Nordic Seas is roughly estimated to be 74 million tons by wet weight (Aksnes & Blindheim, 1996). This copepod has a generation time of 1–2 years in the Norwegian Sea, and has a dormancy period in deep waters during winter. Copepods hatch as nauplius larvae and progress through six naupliar stages and five copepodite stages before reaching sexual maturity. The females spawn at the time of the spring bloom, and the offspring feed to obtain large lipid deposits in summer and autumn (Marshall & Orr, 1972). The storage lipids of late copepodites and adults are in the form of wax esters, which are fatty acids esterified with fatty alcohols. Wax esters are the most common storage lipids in herbivorous zooplankton feeding on short blooms of phytoplankton (Sargent, Gatten, & Henderson, 1981). The profile of the fatty acids in wax esters will reflect the fatty acid composition of the dietary phytoplankton (Lee, Barnett, & Hirota, 1971), and may thereby contain large amounts of *n*-3 fatty acids. The phospholipids mainly consist of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Fraser, Sargent, & Gamble, 1989) and contains a high share of C22:6 *n*-3 and C20:5 *n*-3 (Farkas, Storebakken, & Bhosie, 1988). Due to its fatty acids, *C. finmarchicus* is a promising marine raw material and an interesting lipid source for commercial use.

World aquaculture has grown steadily in the last 50 years, with an annual growth rate of 8.7% (excluding China) since 1970. Meanwhile, for the last 10–15 years most of the world fisheries are fully or overexploited, and the maximum sustainable catch has been reached, of which 20–30% are used for animal feed (FAO, 2008). Further growth in the aquaculture sector requires alternative feed sources. Vegetable sources can replace part of the fishmeal and oil, but the need for *n*-3 highly unsaturated fatty acids (HUFA) like C22:6 *n*-3 and C20:5 *n*-3 will require new marine lipid sources (Turchini, Torstensen, & Ng, 2009). There has also been an increasing awareness of the importance of marine lipids in human nutrition, and beneficial health effects have been documented from consumption of *n*-3 HUFA which are mainly provided by marine lipids. C22:6 *n*-3 and C20:5 *n*-3 are HUFAs that play a vital role in membrane fluidity, cellular signalling, and gene expression. They have been shown to provide positive benefits in coronary heart disease, diabetes, immune response disorders, and mental health (Larsen, Eilertsen, & Elvevoll, 2011).

A rapid change in lipid- and protein composition *postmortem* is found in *C. finmarchicus* (Overrein, 2010), probably caused by digestive enzymes that are present in copepods (Bond, 1934). Phospholipids will be hydrolysed resulting in *lyso*-phospholipids

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and free fatty acids, and *lyso*-phospholipids are further hydrolysed by *lyso*-phospholipases and phospholipases. Proteolytic activity will degrade proteins to peptides and free amino acids. Solgaard, Standal, and Draget (2007), and Solgaard, Thorsen, and Draget (2009) have shown high proteolytic activity in *C. finmarchicus* and also identified a high degree of *postmortem* degradation and subsequent leaching of nutrients as challenges in processing raw material. Similar problems and characteristics are found for other zooplankton species (Grabner, Wieser, & Lackner, 1981), and are also well documented for krill (Ellingsen, 1982; Saether, Ellingsen, & Mohr, 1986, 1987).

To avoid degradation of the raw material, enzymes in *C. finmarchicus* must be inactivated. Heat is the most commonly used denaturing agent in food processing and preservation. From a nutritional standpoint, denaturation of proteins often improves the digestibility and biological availability of essential amino acids (Damodaran, 2008). Enzyme inhibitors and pH alteration are other methods to inactivate enzymes, but for nutritional purposes, a neutral pH would be preferred and inhibitors often have antinutritional effects or are toxic. Drying is also a way to arrest enzymatic activity. However, some enzymes can still be active at low water activities (Parkin, 2008), and the enzymes will be reactivated once the product is rehydrated, as shown in *C. finmarchicus* (Bantle, Eikevik, & Rustad, 2009).

Products with a high content of HUFA are highly susceptible to oxidation during storage. Oxidation results in formation of a variety of volatile compounds giving an unpleasant rancid product; the products formed can also result in possible biological damage. High content of free fatty acids formed by autolytic activity is a parameter for poor quality because they cause off-flavour, reduce oxidative stability and cause foaming (McClements & Decker, 2008). A high content of HUFA gives an even higher oxidation potential. Several strategies can be used to avoid oxidation. Light, heat, oxygen, and pro-oxidants should be avoided to prevent oxidation, while antioxidants will reduce the reaction rate (McClements & Decker, 2008).

The main aim of the present study was to investigate the effect of heat on the activity of proteolytic and lipolytic enzymes in *C. finmarchicus,* and further to evaluate the stability of selected biochemical components during heating and low temperature storage.

2. Material and methods

2.1. Heat treatment experiment

Zooplankton biomass was obtained from two harvests in spring (14th May, 2008) using NTNU's research vessel R/V Gunnerus outside of Frøya off the middle part of the Norwegian coast (63°42′N, 9°00′E). A kite trawl developed at NTNU with plankton nets (mesh size 500 μm) was dragged through the water (1 knot speed) in the surface zone (0–10 m depth) for one hour. Immediately after landing the trawl on deck, the zooplankton was kept on ice and the experiment was started after 30 min. From the zooplankton batch a small sample was fixed in formalin (10%) for identification of species and stages. Ten grams of zooplankton were transferred to 50-mL centrifuge tubes, and boiling fresh water was added giving a total volume of 40 mL in all tubes. The immediate temperature in the mixture was 70 °C, and the tube was incubated in a water bath at 72 ± 2 °C for 5, 10, 15, 20, 25 and 30 min. After heat treatment the samples were immediately placed in ice water. The samples were filtered through a plankton net (mesh size 200 μm) and the two fractions (water and solids) were frozen in preweighed tubes on dry ice (−78.5 °C), which were reweighed on shore. All samples were divided for different biochemical analyses and flushed with

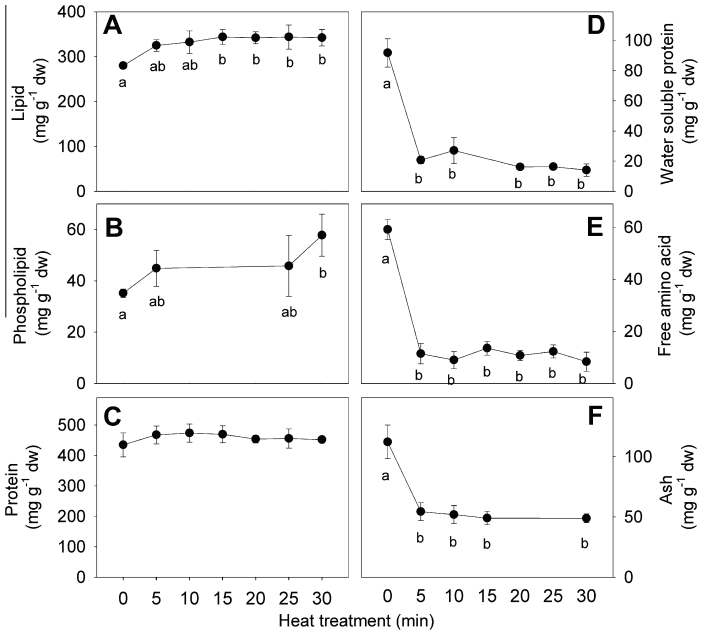

Fig. 1. Biochemical components of the biomass from heat treated *C. finmarchicus* showing total lipid (mg g^{-1} dw) (A), phospholipids (mg g^{-1} dw) (B), crude protein (mg g^{-1} dw) (C), water soluble proteins (mg g^{-1} dw) (D), free amino acids (mg g^{-1} dw) (E) and ash (mg g^{-1} dw) (F). Phospholipids are the sum of PC and PE. Lower case letters imply significant differences between samples.

nitrogen gas before further storage at −80 °C until analysis. Samples for total lipid and fatty acids were in addition freeze-dried (Hetosicc, Heto, Denmark). Heat treatment was performed in triplicates for each holding time, and the whole experiment was repeated (heat treatment 1 and heat treatment 2).

2.2. Heat treatment and long-time storage

Zooplankton biomass was obtained in spring (24th April, 2008) with R/V Gunnerus outside of Frøya, as described above. From the zooplankton batch a small sample was fixed in formalin (10%) for identification of species and stages. The zooplankton was kept on ice (30 min) until heat treatment. Boiling water was mixed with zooplankton (10–15 kg) to give 70 ± 2 °C that was maintained for 15 min with an immersion heater (Elektratherm E2010, GmbH, Lindau, Germany). The solid phase was drained from the water phase with a 500-μm plankton net, cooled down to 30–35 °C (20 min) by spreading the biomass over a large surface, divided in five boxes (15 × 15 × 5 cm) and covered with plastic bags, and frozen on dry ice on board. The core of the biomass in the box took 3 h to reach −20 °C. Samples of untreated zooplankton were also packed and frozen as described above. On shore, the boxes were placed in a freezer (−20 °C) and kept there for 4, 6, 8, 10 and 12 months. Samples were then kept at −80 °C until analysis.

2.3. Analytical methods

2.3.1. Dry weight, ash and development stages

To obtain dry weight of the samples, three tubes with 10 mL zooplankton were frozen in dry ice on board and weighed in the laboratory at shore. Dry weight was calculated from drying (105 °C) for minimum 24 h, until stable weight. Ash was calculated after heating (600 °C) for 12 h in an oven (Hagan Elektroovner A/S, Oslo, Norway). *C. finmarchicus* and its developmental stages were determined under a dissecting microscope, based on morphological criteria (Mauchline, 1998) and size (Unstad & Tande, 1991), as the average of two samples of at least 70 individuals.

2.3.2. Lipid and astaxanthin analysis

Total lipids were extracted and determined gravimetrically according to Bligh and Dyer (1959) with modifications described by Jakobsen, Aasen, Josefsen, and Strom (2008), and analysed in duplicates. Fatty acid methyl esters from extracted lipid were prepared according to Metcalfe and Schmitz (1961), and analysed with a gas chromatograph (AutoSystem XL, Perkin Elme, Waltham, MA) with TotalChrom Version 6.3.1 software. The system was equipped with an auto-injector (injection volume of 1 μL, on-column injection, inlet temperature 250 °C) and a flame ionisation detector (FID, 280 °C). A fused silica capillary column coated with a chemically bonded polyethylene glycol (CP-Wax 52CB, 25 m × 0.25 mm i.d.; Varian, Palo Alto, CA) was used. The temperature program for the oven was 90 °C for 1 min, then raised to 150 °C at 30 °C min^{-1} and finally raised to 225 °C at 3 °C min^{-1} and held for 7 min. Helium was used as the carrier gas. The retention times of the fatty acid methyl esters were compared to commercial standards (Nu-Chek Prep, Tokyo, Japan) and quantified by the use of C19:0 as an internal standard added prior to extraction in combination with external standard curves. Total lipid was expressed in mg per g of dry weight (dw).

Lipid classes were analysed with thin-layer chromatography coupled with a flame ionisation detector (TLC-FID, Iatroscan MK 6; SES GmbH, Bechenheim, Germany). The detector was operated with an air flow of 2000 mL min^{-1} and a hydrogen flow rate of 160 mL min^{-1}. Thin quartz rods coated with silica (Chromarods SIII) were used to separate lipid classes. Lipids extracted as described were diluted in chloroform and 1 μL was applied to the rods with a 5-μL syringe. The rods were developed in two different solvent systems. For neutral lipid classes hexane:diethyl-ether:formic acid (85:15:0.04, v:v:v) was used, as described by Fraser, Tocher, and Sargent (1985). For polar lipid classes chloroform: methanol: water (67:30:3, v:v:v) was used. The rods were held in a chamber with saturated NaCl (Merck, Germany) solution for 8 min and developed in the solvent system for 27 min. They were then dried for 3 min in hot air before burning the rods (FID) to obtain a chromatogram. The scanning speed was set at 30 s rod^{-1}. Data were collected and integrated with SES ChromStar Version 6.3. The retention times of the lipid classes were compared with commercial lipid standards and quantified with external standard curves. Natural soy PC and PE (Avanti Polar Lipid, Alabaster, AL), and oleic acid (Nu-Chek Prep) were used to make five point standard curves ($r^2 > 0.99$). All samples were analysed in duplicates with four or five rods per replicate and expressed in mg per g dw.

Astaxanthin was extracted as described by Bligh and Dyer (1959). The chloroform was evaporated and the lipid phase was dissolved in acetone, and determined with a Varian Cary 50 spectrophotometer as described by Foss, Storebakken, Schiedt, Liaaen Jensen, Austreng and Streiff (1984). Total astaxanthin was expressed as mg per kg dw.

2.3.3. Enzymatic analysis

Phospholipase A_2 (PLA_2) activity was determined with a phospholipase assay kit (Cayman Chemical, Company, Ann Arbor, MI) and a Varian Cary 50 spectrophotometer. The analysis was performed as described in the kit, where arachidonoyl thio-phosphatidylcholine was the substrate for total PLA_2 activity. The frozen biomass was homogenised in HEPES buffer (50 mM, pH 7.4), 1 part biomass and 1 part buffer (w:v), in 2.5-mL tubes containing 1.4-mm zirconium oxide beads (Bertin Technologies, Saint-Quentin en Yveline, France) with a Precellys homogeniser (5000 rpm, 20 s). The samples were centrifuged (10,000*g*, 15 min, 4 °C) and the supernatant was collected and kept frozen (−20 °C) until analysis. PLA_2 activity was expressed as μmol enzyme hydrolysing arachidonoyl thio-phosphatidylcholine per min at 25 °C per g ww of biomass.

Water extract for determination of proteolytic and lipolytic activity were prepared by extracting *C. finmarchicus* with distilled water, 1 part biomass to 2 parts water (w/v). The mixture was homogenised with an Ultra-Turrax for 20 s and centrifuged (10,400*g*, 20 min, 4 °C, Sorvall RC-5B, DuPont Instruments). The water extract was filtered through glass wool and the volume of the extract was determined.

General proteolytic activity was determined as described by Stoknes, Rustad, and Mohr (1993) using haemoglobin (1% (w/v)) as substrate, and expressed as increase in amount of acid-soluble peptides (mg cut haemoglobin per mg soluble protein) per minute. The samples were analysed in triplicate. The extracts were mixed with phosphate-citric acid buffer (McIlvaine, 1921) at pH 7 and incubated in a water bath at temperatures between 30 and 70 °C. Incubation at 20 °C took place in the laboratory (room temperature) and incubation at 5 °C took place in a temperature-controlled room. After incubation, the proteins in the samples were precipitated with trichloroacetic acid (TCA) (final concentration 2.5%) and the content of acid soluble peptides was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

Lipolytic activity was determined by spectrofluorimetry as described by Roberts (1985) and later by Izquierdo and Henderson (1998), with minor modifications. The non-fluorescent substrate 4-methylumbelliferyl heptanoate was dissolved in a liposomal dispersion of soy lecithin. The lipases hydrolysed the substrate to heptanoic acid and the strongly fluorescent compound, 4-methylumbelliferone. Mixtures of 20 μL substrate and 40 μL enzyme solution, suitably diluted in phosphate-citric acid buffer (McIlvaine,

Table 1
Data from time of harvest, temperature of heat treatment and relative amounts of different developmental stages in samples used for heat treatment and long-time storage. Development stages are given as copepodite stage 4 (CIV), copepodite stage 5 (CV), female (F) and male (M).

	Long time storage	Heat treatment 1	Heat treatment 2
Start place, trawl	63°50′N, 8°49′E	63°42′N, 9°00′E	63°42′N, 9°00′E
Time of day	24th April, 11:20	14th May, 09:30	14th May, 15:00
Temperature of heat treatment	70 °C	74 °C	70 °C
Amount of stages (% in numbers) CIV:CV:F:M	77:23:0:1	10:62:21:9	12:74:10:4

1921) pH 5 or 7, were incubated for 15 min in a water bath at 40 °C. The reaction was stopped by adding 3 mL cold 1 M Tris HCl, pH 7.5. Zero samples were incubated in a water bath at 80 °C for 30 min, centrifuged at 420*g* for 10 min, and diluted and read in the same way as the samples. The increase in emission at 450 nm (excitation at 365 nm) was determined using a Perkin Elmer 3000 spectrofluorimeter. The activity was expressed as an increase in fluorescence and is given in arbitrary units (U) based on the average of three readings.

2.3.4. Amino acids, TCA solubles and protein analysis

The amount of free amino acids was determined in crude enzyme extracts from biomass and in the water phase after precipitating the proteins in sulfosalicylic acid (2% final concentration) and diluting the supernatant with deionised water, as described by Osnes and Mohr (1985). Reverse-phase HPLC, by precolumn derivatisation with o-phthaldialdehyde, was performed using a NovaPak C18 cartridge (Waters, Milford, MA) (Flynn, 1988). The amount of TCA soluble peptides was determined in crude enzyme extracts from biomass and in the water phase after precipitation with 10% TCA as described by Rohm, Jaros, Rockenbauer, Riedler-Hellrigl, Uniacke-Lowe and Fox (1996), except the samples were kept at room temperature for 30 min instead of overnight at 4 °C. The contents of TCA solubles and proteins in the extracts were determined by the Lowry method using bovine serum albumin (Sigma–Aldrich, A9647) as a standard.

The content of carbon and nitrogen in freeze dried zooplankton was determined with an element combustion system (Costech Analytical Technologies Inc., Valencia, CA). Crude protein was calculated from the content of total N by the conversion factor 4.94 (Salo-Väänänen & Koivistoinen, 1996), as they showed large deviations in nitrogen-to-protein conversion factors between different food groups.

2.4. Statistical methods

Normality of data was tested using Kolmogorov–Smirnov, and homogeneity of variances was tested using the Levene's statistics. Samples were compared by using one-way ANOVA with the posthoc test Games-Howell that compensates for uneven variance and low *n*. SPSS Statistics 17.0 was used for all the statistical calculations. Data are presented as means with standard deviations.

3. Results

Harvest samples of marine zooplankton were obtained during spring bloom (24th April and 14th May) off the middle of the Norwegian coast, and consisted of 99% *C. finmarchicus*. The trawl (mesh size, 500 μm) retained copepodite stage 4 (CIV), stage 5 (CV), females and males, and the distribution is shown in Table 1.

3.1. Heat treatment

The activity of selected autolytic enzymes was measured before heating was applied and during the 30 min of heat treatment. In untreated biomass of *C. finmarchicus* there was proteolytic activity (1.7 ± 0.3 increase in amount of cut protein in % ww), lipolytic activity ($2.0 \times 10^4 \pm 0.2 \times 10^4$ increase in fluorescence) and PLA_2 activity (17 ± 1 mmol min^{-1} g^{-1} (ww)). No activity in the selected enzymes could be detected after 5 min of heat treatment at 72 ± 2 °C.

The total lipid content of the biomass was on average 34 ± 2% dw after heat treatment, and most of the lipids were retained during heat treatment (Fig. 1A). The content of phospholipids (Fig. 1B) and *n*-3 fatty acids in the biomass were not influenced by the hot water treatment and the *n*-3 fatty acids were on average 65 ± 6 mg g^{-1} dw after heat treatment. The amount of crude protein was unchanged after heat treatment and constituted 46 ± 3% dw (Fig. 1C). However, a decreased content of water soluble proteins was found in the biomass (Fig. 1D) with a reduction from 92 mg g^{-1} dw before heat treatment to an average of 19 mg g^{-1} dw after heat treatment, giving a reduction of 80%. From mass balances of the water phases and biomass we can see that 55% of the water soluble proteins were denatured. Of the 45% water soluble proteins left, 35% was in the biomass and 65% in the water phase. After heat treatment, most of the content of free amino acids (Fig. 1E) and TCA solubles was found in the water phase. The biomass contained on average 6 ± 2 mg g^{-1} dw TCA solubles and 11 ± 2 mg g^{-1} dw amino acids after heat treatment.

By mixing the biomass with fresh water, the salt was washed out and the ash content was thereby reduced from 112 ± 14 to 51 ± 7 mg g^{-1} dw (Fig. 1F). The loss of water soluble proteins, TCA solubles, free amino acids and salt into the heating water gave an average reduction of 15% of the dry weight.

3.2. Frozen storage

Heat-treated and untreated biomass was stored for 12 months, and several biochemical components were analysed during the storage. Untreated biomass stored at −20 °C for 12 months led to a loss of all phospholipids, which were reduced to zero before 4 months of storage (Fig. 2A). The same treatment showed increased content of FFA and reached a stable content of 36 ± 2 mg g^{-1} dw after 6 months (Fig. 2B). Heat treatment (70 ± 2 °C, 15 min) of *C. finmarchicus* resulted in a stable content of phospholipids (61 ± 3 mg g^{-1} dw) after 12 months of storage, and formation of FFA was low and stable (2.8 ± 0.5% of total lipid). The content of lipids was stable in both untreated and heat treated material (Fig. 2C). The content of ash in the samples was 116 ± 7 mg g^{-1} dw in the untreated material and 41 ± 3 mg g^{-1} dw in material from the hot water treatment.

The content of total astaxanthin in untreated *C. finmarchicus* was unchanged during storage (355 ± 26 μg g^{-1} dw) (Fig. 2D). Similarly, storage of heat treated biomass did not alter the astaxanthin content (535 ± 26 μg g^{-1} dw). The *n*-3 fatty acids were unchanged after 12 months of storage in both treatments and were on average 64 ± 6 mg g^{-1} dw (Fig. 2E).

4. Discussion

4.1. Heat treatment

Heat treatment of *C. finmarchicus* for 5 min at 70 °C inactivated all tested proteolytic activity. An optimum temperature of 50 °C has been found for proteolytic activity in extracts of *C. finmarchicus* (Solgaard et al., 2007), which is similar to that found for Antarctic

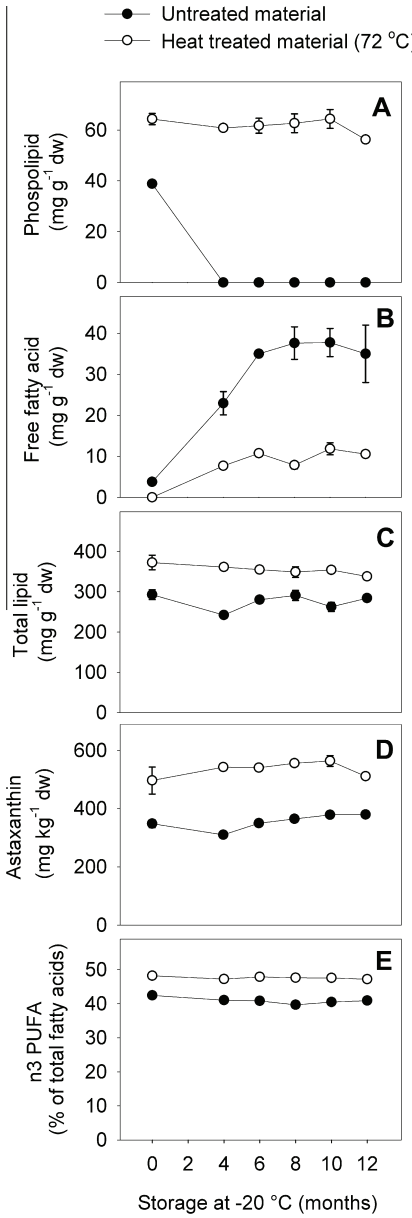

Fig. 2. Biochemical components from long time stored *C. finmarchicus* both heat treated (72 ± 2 °C) (open dots) and untreated (closed dots) showing phospholipids (mg g^{-1} dw) (A), free fatty acids (mg g^{-1} dw) (B), total lipid (mg g^{-1} dw) (C), astaxanthin (mg kg^{-1} dw) (D), and n3 fatty acids (% of total fatty acids) (E). Phospholipids are the sum of PC and PE.

krill (40–50 °C) (Kolakowski & Sikorski, 2000; Osnes & Mohr, 1985). Kolakowski and Sikorski (2000) concluded that the only way to stop autolysis completely in krill is by boiling. However, heat treatments that includes a long holding time around 50 °C should be avoided, if the requirement is a low content of free amino acids and peptides. As shown by Overrein (2010) holding *C. finmarchicus* at 40, 50, and 60 °C gave an increased amount of TCA solubles and free amino acids. Heat treatment at 70 °C reduced the amount of peptides and free amino acids, but the content was still higher than in the zero sample and was probably caused by a 7 min heating time from 0 to 70 °C. In the present study the temperature was increased from 0 to 70 °C instantly by mixing *C. finmarchicus* with boiling water. However, by mixing with water, most of the water-soluble peptides and amino acids were released to the water phase. Heat will primarily affect the stability of non-covalent interactions in proteins. Hydrogen bonding and electrostatic interactions are destabilised with increasing temperature while hydrophobic interactions are stabilising, until 70–80 °C. When enough kinetic energy is applied, the protein unfolds, aggregates, and precipitates, giving a decreased solubility. Denaturation temperatures of enzymes vary and depend, among other factors, on the amino acid composition and environmental factors (Damodaran, 2008).

Heat treatment of *C. finmarchicus* for 5 min at 70 °C inactivated all tested lipolytic and phospholipase activity. The phospholipids were maintained in the heat treatment, also showing that the inactivation of enzymes was sufficient. Overrein (2010) showed a decreased level of phospholipids in heat treatments of *C. finmarchicus* at 40, 50 and 60 °C, indicating enzymatic activity. The thermal stability of krill lipase is found to be relatively poor and it was inactivated at 60 °C (Kolakowski & Sikorski, 2000). Phospholipids are rapidly hydrolysed in krill and *C. finmarchicus*, compared to the neutral lipid classes (Ellingsen, 1982; Overrein, 2010; Saether et al., 1986). It was expected that more lipids should be released in the water phase from 70 °C heat treatment, caused by the easily breakable oil sac of *C. finmarchicus*. Per dry weight the content of lipid was higher after heat treatment, but this was caused by reduced amounts of salt and other water soluble components, which reduced the dry weight. Some oil droplets, however, were observed in the liquid phase.

The untreated biomass showed proteolytic, lipase and PLA_2 activity, and the enzymes present will vary through the day, through the harvesting season, and between biomass from different geographical locations (Tande & Slagstad, 1982). Digestive enzymes are regulated depending on which food is present, as found in *C. helgolandicus* (Harris, Samain, Moal, Martinjezequel, & Poulet, 1986), and this could result in different rates of autolysis. Enzymes could also originate from the food of copepods present in the digestive system, giving a contribution to autolysis. However, it is unlikely that the stability of the enzymes will be affected by these variations.

4.2. Frozen storage

Increased levels of FFA were found in the untreated *C. finmarchicus* during long time storage at −20 °C. Lipolytic activity in fish muscle during cold storage has long been recognised as a challenge, resulting in quality deterioration. Lovern and Olley (1962) showed the highest free fatty acid accumulation at −4 °C in cod flesh, and activity has been observed even at −70 °C (Brockerhoff, 1974). In krill stored at −20 °C, an increased level of free fatty acid (39–53 % of total lipid) and a decreased level of phospholipids were found (Kolakowski & Sikorski, 2000). Overrein (2010) showed an increased level of FFA in *C. finmarchicus* stored at 0 °C and the same level as found in this study (3.5% dw) was observed after 24 h at 12–14 °C. An increased level of FFA makes the biomass more susceptible to oxidation, since FFAs are more easily oxidised than when they are attached to other molecules (wax esters, triacylglycerols and phospholipids), and the high content of HUFA gives an even higher oxidation potential. Lipid oxidation starts with the forming of a fatty acid radical, and the potential for this formation increases with increasing unsaturation (McClements & Decker, 2008). The amount of HUFAs could thus be used as an indicator of the oxidative status during storage. In this study the content

of *n*-3 HUFAs was stable in the stored biomass. The content of astaxanthin in the stored biomass was maintained after 12 months frozen storage. Astaxanthin is an antioxidant, meaning that it is capable of inhibiting the oxidation of other molecules, and is also a valuable feed ingredient in salmonid and crustacean aquaculture. A maintained content of astaxanthin could imply a stable biomass with regard to oxidation. Analysis of primary and secondary oxidation products would be more sensitive and give a more precise measure of the oxidation status, but was not performed in the present study.

Wax esters seem to be fairly resistant towards autolysis in *C. finmarchicus*. In this study, the amount of wax esters did not decline during storage, since the amount of free fatty acids is explained by the degradation of phospholipids. Results are similar to those of Overrein (2010), when biomass was stored at 0 °C. Also in krill that contains wax esters, no reduction was found during storage at 0 °C (Ellingsen, 1982; Saether et al., 1986). Wax esters are rarely found in the food of copepods and they may not need a wax ester hydrolase in their digestive system. However, for their catabolism they need this enzyme to mobilise wax esters for energy. Sargent and Henderson (1986) suggest that the hydrolysis of wax esters in calanoid copepods involves a specific and hormone-sensitive lipase. Krill on the other hand feed on zooplankton that contains wax esters and should have the enzymatic apparatus to digest wax esters rapidly. Kolakowski and Sikorski (2000) suggest that the rapid degradation of phospholipids compared to neutral lipids is a result of a higher phospholipase activity compared to other lipolytic activities. However, it could be caused by improved contact of the enzyme to the more hydrophilic phospholipids, as suggested by Ellingsen (1982). Wax esters in *C. finmarchicus* are physically separated in an oil sac and could be the reason for a poor enzyme to substrate contact in addition to the highly hydrophobic character of wax esters.

Wax esters are the main lipid class in *C. finmarchicus*; Atlantic salmon (*Salmo salar*) is able to utilise *Calanus* oil in their diets (Olsen et al., 2004) demonstrating the suitability of wax esters from *C. finmarchicus* as a new marine ingredient in fish feed. Wax esters from *C. finmarchicus* have a low melting point and long-chain, monounsaturated fatty alcohols, which makes them interesting in cosmetics, lubricants, and pharmaceuticals, as discussed by Sargent and Henderson (1986). Phospholipids in *C. finmarchicus* have high shares of C22:6 *n*-3 and C20:5 *n*-3, and could suit dietary demands for early marine fish larvae, as they require phospholipids (Tocher, Bendiksen, Campbell, & Bell, 2008) in addition to a high content of C22:6 *n*-3 and C20:5 *n*-3 (Sargent, Tocher, & Bell, 2002). Marine proteins will decompose to peptides and free amino acids if the autolytic enzymes are active. A raised content of peptides and free amino acids in feed, gives a positive effect in growth or survival to some marine species (Kolkovski, 2001). However, the advantage of a high content of proteins could be more important.

When harvesting marine biomass in the future, the aim should be to utilise as much as possible, for the aquaculture industry, as nutraceuticals or as fine chemicals. The need for inactivation of enzymes in the biomass is necessary. Heating is a convenient method to inactivate enzymes responsible for autolysis, and developments to optimise heating procedures and equipment on board the vessel are needed.

5. Conclusion

To maintain the content of phospholipids and proteins in harvested biomass, it should be heat treated on board the vessel directly after harvest. Instant heating of *C. finmarchicus* is a convenient way to inactivate autolytic enzymes and 5-min holding time at 72 ± 2 °C is sufficient to stop degradation of phospholipids and proteins. By using fresh water for heat treatment, water-soluble proteins and free amino acids were released to the water phase, and the salt concentration was significantly reduced. Phospholipids in heat-treated *C. finmarchicus* were maintained upon one year of frozen storage (−20 °C), whereas storage of untreated biomass under the same conditions led to a loss of all phospholipids before 4 months of storage.

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Paper IV

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LIPID CLASS CHANGES AND LIPID OXIDATION IN *CALANUS FINMARCHICUS* DURING VACUUM, ATMOSPHERIC AND NITROGEN FREEZE DRYING

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Abstract: Different freeze drying processes (vacuum, atmospheric and nitrogen) were evaluated as preservation methods for the zooplankton species *Calanus finmarchicus*. Changes in lipid composition and lipid oxidation were evaluated for the dried products. The drying rate was highest for atmospheric freeze drying and lowest for nitrogen freeze drying. Nitrogen freeze drying resulted in a reduced amount of phospholipids (92 % loss) and a free fatty acid content of 4.7 % (of dry matter), whereas atmospheric freeze drying preserved the lipids in a quality similar to vacuum freeze drying (57 % loss of phospholipids, and 1.7 and 2.9 % free fatty acids of dry matter).

Keywords: zooplankton, *Calanus finmarchicus*, freeze drying, marine lipids, degradation

INTRODUCTION

Freeze drying is generally employed with food, pharmaceutical and certain biological materials that need to be processed in a frozen condition. The product is dehydrated by sublimation of the frozen water in the product and removal of the vapor. This results in a porous and non-shrunken product structure with good rehydration characteristics and little loss of flavor and aroma. Since no liquid water is present in freeze drying, the product is locally dehydrated very rapidly, which reduces degradation due to enzymatic reactions, protein and lipid degradation and non-enzymatic browning. Freeze drying is increasingly used for dehydrating food such as coffee, certain sea foods, fruits and vegetables (Mujumdar, 2007). Freeze drying is mostly carried out under vacuum at temperatures of -10°C or lower and at pressures lower than 300Pa (VFD). The product is frozen by low temperature cooling, dried by direct sublimation and stored in a dry state under controlled conditions. Due to the use of vacuum and the slow sublimation rates, freeze drying is generally an expensive process.

Recent developments in heat pump technology have made it possible to freeze-dry products at ambient pressures (AFD), where up to 60-80% of the energy can be recovered by circulating and conditioning of the drying air (Strømmen et al., 2002; Colak and Hepbasli, 2009). As shown by Bantle et al. (2009) and Di Matteo et al. (2003), drying rates in AFD can be significantly faster than in VFD when the product is dried as small particles, which improves heat and mass transfer with the drying agent. AFD is normally undertaken at temperatures between -5 °C and -10 °C, where the vapor pressure of water is between 400Pa and 250Pa respectively. It is also possible to dry at temperatures closer to the initial freezing point of the product, where the vapor pressure and sublimation rate are at their maxima. AFD is generally carried out with ambient air as the drying agent, which moderates investment costs. In the case of products that are highly sensitive to oxygen, the drying processes can also be undertaken with inert drying agents (Hawlader et al., 2006). This is also possible in freeze drying when the ambient air can be removed by an inert gas, e.g. nitrogen (NFD).

World aquaculture has grown dramatically in the last 50 years, and has an annual growth rate of 8.7 percent (excluding China) worldwide since 1970. Meanwhile, the world marine fisheries have been overexploited and depleted for the last 10 – 15 years, and the maximum wild capture fisheries potential has probably been reached. (FAO, 2009). Fish meal and fish oil are important ingredients in aquaculture feed as they supply essential amino acids and fatty acids as docohexaenoic acid (DHA) and eicosahexaenoic acid (EPA). If aquaculture continues to grow, alternative feed resources are required. (Naylor et al., 2000). Vegetable sources are most likely to replace parts of the meal and oil because of its availability and low cost. However, the need for n-3 poly unsaturated fatty acids (PUFA) will require a marine

source of lipids (Turchini et al., 2009). Also in human nutrition, there has been an increasing awareness of the importance of marine lipids. Many beneficial health effects have been documented from consumption of the omega-3 PUFA, which are mainly provided by marine lipids. DHA (C22:6 n3) and EPA (C20:5 n3) are PUFAs that play a vital role in membrane fluidity, cellular signaling, gene expression, and eicosanoid metabolism. They have been shown to provide positive benefits in coronary heart disease, diabetes, immune response disorders, and mental health (Simopoulos, 1991).

Calanus finmarchicus (CF) is the dominant zooplankton species in the Norwegian and Barents Seas (Planque and Batten, 2000). The annual production of CF in the Nordic Seas is roughly estimated to be 74 million tons by wet weight (Aksnes and Blindheim, 1996). Its high amount of marine lipids includes mainly wax esters (fatty acids esterified with fatty alcohols) and phospholipids, and it contains high amounts of omega-3 long-chained PUFAs, such as DHA and EPA. It also contains high amounts of the omega-3 fatty acid, stearidonic acid (SDA, C18:4 n3) (Sargent and Henderson, 1986). The phospholipids in CF consist mainly of phosphatidyl choline (PC) and phosphatidyl etnanolamine (PE) (Fraser et al., 1989). This makes CF a promising marine resource for proteins and lipids. However, when these highly unsaturated lipids are exposed to air, they give rise to a variety of volatile compounds resulting in an unpleasant rancid product. The oxidation products can also result in possible biological damage. Enzymatic degradation of lipids and proteins leads to formation of free fatty acids, peptides and amino acids. Free fatty acids are more easily oxidized than triacylglycerols, phospholipids and wax esters. Several strategies can be used to avoid oxidation and enzymatic activity. Light, heat, oxygen, and pro-oxidants should be avoided to prevent oxidation, while antioxidants will lower the speed of reactions. Water and temperature have a great influence on enzyme activity (Frankel, 1998; Damodaran et al., 2008).

The current endeavor is to catch and use CF at an industrial scale. Overrein (2010) has shown a high change in lipid- and protein composition *post mortem* due to autolytic enzymes. Solgaard et al. (2007) and Solgaard (2008) have shown the high proteolytic activity of CF and also identified the high degree of *post mortem* degradation and subsequent leaching of nutrients as challenges in processing CF. Grabner et al. (1981) documented similar problems and characteristics for other zooplankton species.

CF is therefore a prime candidate for the use of freeze drying as a dehydration method to stabilize and preserve its proteins and lipids.

For this study, the amount of lipid, PUFA, phospholipids, free fatty acids and lipid oxidation of CF were investigated during freeze drying under vacuum (VFD) and ambient conditions (AFD) as a quality measure. Since the amount and composition of lipids in CF also indicated a high lipid oxidation potential, nitrogen was used as the inert drying agent in an additional freeze drying process (NFD). Lipid changes during the different freeze drying processes were investigated.

MATERIALS AND METHODS

Calanus finmarchicus

Zooplankton can be harvested during the spring season, when their concentration close to the ocean surface is on the increase. A plankton trawl with a 500µm inner net was used to catch industrial-sized batches of CF between the islands of Hitra and Frøya (GPS: 63º30N, 9º55E) outside of Trondheim Fjord, Norway at the end of April 2009. The catch consisted mostly of CF at stages four and five, which is in accordance with Tokle (2006). It was immediately frozen in 0.5cm plates, vacuum-packed and stored on dry ice. The CF plates were stored at -80°C in the laboratory until further processing. Twenty-four hours prior to freeze drying, the CF plates were stored in a -10°C freezing room in order to improve their physical properties before crushing. CF was then crushed to a particle size of d_{50}=2mm with an industrial meat grinder, which was also tempered at -10°C. The bulk of CF was then split into 3 batches and dried using VFD, AFD, and NFD.

Freeze drying

VFD was carried out using an "Alpha 1-4 LD" (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz/Germany) vacuum freeze dryer with an ice condenser temperature of -60ºC. A vacuum of 0.0014 bar was applied, which gives a drying temperature of ≈-17ºC.

Crushed CF (500g) was placed in a neck filter bottle and connected to the vacuum chamber. Weight reduction was determined manually, by disconnecting and weighing the bottle. The VFD was placed in the laboratory at ambient conditions (≈20ºC), so the heat for sublimation was conducted through the sample bottle into the bulk of CF.

For AFD, we used a system as shown in Figure 1 , which allows a SMER (specific moisture extraction ratio) of up to 4 kg_{water} kWh^{-1} (Colak and Hepbasli, 2009). The product was dried in fluidized state, about 10-20% over the minimum fluidization velocity. The drying air was circulated at the condenser and evaporator of a heat pump system. The temperature and humidity of the drying air decreases at the cold surfaces of the evaporator, where the condensation energy is transferred to the refrigerant. The dry air is then heated up again in the condenser of the heat pump system, thereby regaining its own energy. For this study, the temperature in the evaporator was -

30ºC and the condenser temperature was -4ºC. The drying air was therefore conditioned to -6ºC (±0.3K) and 22% (±5%) relative humidity. The removable drying chamber was sealed with a 1mm perforated plate at the bottom and a 300µm mesh at the top. To determine the sublimation rate, the drying process was interrupted at specific time intervals and the weight reduction of the drying chamber, including the product, was determined manually. Crushed CF (1000g) was dried in fluidized bed state during each AFD experiment.

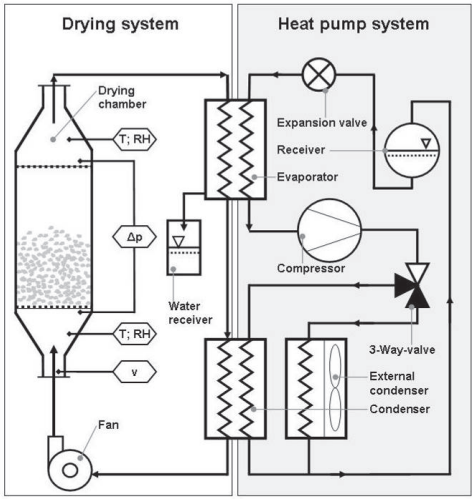

Fig. 1. Heat pump assisted atmospheric freeze drying in a fluidized bed

Nitrogen was used as an inert drying agent in a separate drying chamber (NFD), because it was impossible to replace all the oxygen in the AFD system. Pure nitrogen gas (99.9%) was stored in compressed bottles at -8ºC (±1K) in the freezing room along with the drying chamber. The bottles were connected below the bottom plate of the drying chamber. There was no measurable oxygen content in the drying chamber when the gas bottles were open and the relative humidity of the nitrogen was below the hygrometer's sensitivity of 3%. The moisturized nitrogen was not circulated and was discharged directly to the ambient air. The drying rate was determined by interrupting the drying process, closing the valves, disconnecting the drying chamber, and manually determining the weight reduction in the chamber, including the product. Around 100g of crushed CF was dried in a stationary bed state in each NFD experiment.

Three freeze drying experiments were performed using AFD and VFD and two using NFD. The moisture content of the product before and after drying was determined from the weight reduction of the sample after heating for 24h at 105ºC.

Lipid analysis

Total lipids were determined gravimetrically as described by Bligh and Dyer (1959). All samples were analyzed in triplicates.

Fatty acid methyl esters were prepared according to Metcalfe et al. (1961) and analyzed with a gas chromatograph (Perkin Elmer AutoSystem XL) with TotalChrom. The system was equipped with an auto injector (injection volume of 1 µl, on-column injection, inlet temperature 250 ºC) and a flame ionization detector (FID, 280 ºC). A fused silica capillary column (Varian, 25 m long, 0.25 mm inner diameter) coated with a chemically bonded polyethylene glycol (CP-wax 52CB) was used. The temperature program for the oven was 90ºC for 1 minute, which was then raised to 150ºC at 30ºC/min and finally raised to 225ºC at 3ºC/min and held for 7 minutes. Helium was used as the carrier gas. The retention times of the fatty acid methyl esters were compared to commercial standards (Nu-Chek Prep) and quantified by the use of C19:0 as an internal standard (Nu-Chek Prep) in combination with external standard curves. All samples were analyzed in triplicates.

Lipid classes were analyzed with thin layer chromatography coupled with a flame ionization detector (TLC-FID, Iatroscan MK 6). The detector was operated with an air flow of 2000 ml/min and a hydrogen flow rate of 160 ml/min. Thin quartz rods coated with silica (Chromarods SIII) were used to separate lipid classes. Lipids that were extracted as described were diluted in chloroform and 1µl was applied to the rods with a 5µl syringe (Hamilton). The rods were developed in two different solvent systems. For neutral lipid classes hexane:diethyl-ether:formic acid (85:15:0.04) was used, as described by Fraser et al. (1985). For polar lipid classes chloroform: methanol: water (67:30:3) was used. The rods were held in a chamber with saturated NaCl solution for 8 minutes and developed for 27 minutes. They were then dried for 3 minutes with hot air before burning the rods in the FID to obtain a chromatogram. The scan speed was set at 30sec/rod. Data were collected and integrated with ChromStar. The retention times of the lipid classes were compared with commercial lipid standards (Nu-Chek Prep) and quantified with external standard curves. Natural soy PC and PE (Avanti Polar Lipid) and oleic acid (Nu-Chek Prep) were used to make standard curves. All samples were analyzed in du- or triplicates with four or five rods per replicate.

Lipid oxidation was measured as concentration of secondary oxidation products, thiobarbituric acid reactive compounds (TBARS). TBARS values were determined by the spectrophotometric method as described by Ke and Woyewoda (1979) and analyzed in duplicates. The absorbance values of samples were compared to a standard curve prepared with 1,1,3,3-

tetraethoxypropane for the calculation of TBARS concentrations (μM/g lipid).

RESULTS

The water content of CF before drying was 82% (wet weight basis). Table 1 gives the average final water content from the drying experiments and the mass transfer rate averaged over the total drying time.

Table1. Final moisture content and drying rates for AFD, VFD and NFD

Freeze drying technique	Moisture $\%_{w.w.}$	Drying rate, $kg_{H_2O} kg_{d.m.}^{-1} h^{-1}$
Atmospheric (AFD)	12.7	0.22
Vacuum (VFD)	8.4	0.10
Nitrogen (NFD)	20.0	0.04

AFD showed the highest drying rate and the product reached its final moisture content after 24 hours. VFD needed around 48-60 hours until no further weight reduction was observed. NFD showed the lowest drying rate, in which the product reached a high but stable water content after 120-140 hours (5-6 days). A higher mass flow of drying agent and a reduced product weight in the drying chamber did not increase the drying rate in NFD.

The total lipid in the dried material was 16.4 ± 0.9, 15.5 ± 0.7 and 14.9 ± 0.3 % of dry weight for AFD, VFD and NFD respectively. Before drying, the lipid content was 15.9 ± 0.2 % of dry weight. The total lipid content decreased linearly with the drying rate (Fig. 2A). Free fatty acids were 17 ± 4, 29 ± 15, 47 ± 10 mg/g d.m. in AFD, VFD and NFD respectively and accounts for 10, 19 and 32 % of the total lipid. This gives a linear relationship between drying rate and free fatty acid content as shown in Fig. 2A.

Phospholipids in CF and in the dried samples consisted mainly of PC and PE. Fig. 2B shows that the content was similar for PC and PE in AFD and VFD (1.0 ± 0.3 and 1.0 ± 0.5 mg PC/ g d.m., and 0.7 ± 0.2 and 0.6 ± 0.3 mg PE/g d.m. in AFD and VFD respectively). For NFD, the content of PC and PE was about 0.1 mg/g d.m. There is a small decline in both PC and PE when drying rate goes down. Before drying, the PC and PE content was 22 ± 3 and 15 ± 2 mg/g dry matter. In AFD and VFD the phospholipid content is more than halved (57 %) compared to the original amount, and in NFD 92 % of phospholipids are lost.

A linear relation was also found for FFA and phospholipids (Fig. 3.).

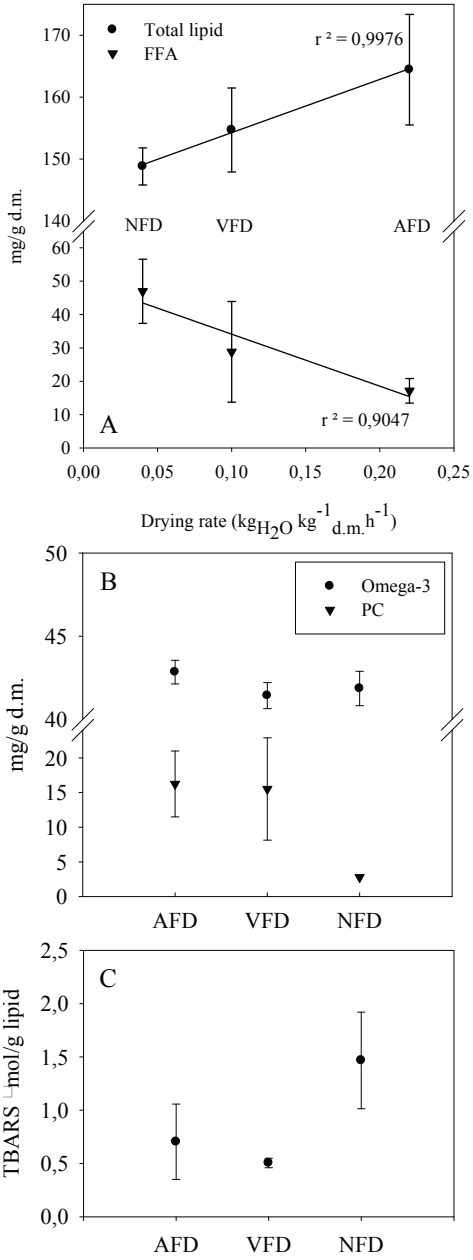

Fig. 2A. Linear regression curve for drying rate ($kg_{H2O}kg^{-1}{}_{d.m.}h^{-1}$) and total lipid (mg/g d.m.), and FFA (mg/g d.m.) for dried CF from AFD, VFD and NFD. Fig.2B. Content of omega-3 fatty acids and phospholipids PC and PE in dried CF from AFD, VFD and NFD (mg/g d.m.). Fig. 2C. Secondary oxidation products determined as TBARS (μmol/glipid) for dried CF from AFD, VFD and NFD. Values are given as averages ±stdev

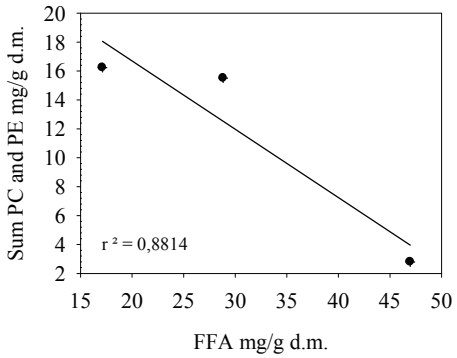

Fig. 3. Linear relation for FFA and phospholipids represented as the sum of PC and PE mg/g d.m. in dried CF from AFD, VFD and NFD. Values are given as averages.

The fatty acid composition was measured and there were no differences in the amount of omega-3 fatty acids among the samples (Fig. 2B). No differences were found in the other fatty acids either (Table 2). Omega-3 fatty acids in the dried samples consist of DHA (C22:6 n3), EPA (C20:5 n3), stearidonic acid (SDA, C18:4 n3) and small amounts of C18:3 n3 and C20:4 n3. They comprise 60 % of the total fatty acids.

Table 2. Fatty acid composition for dried CF from AFD, VFD and NFD (mg/g d.m.). Values are given as averages ± stdev.

Fatty acids	AFD (mg/g d.m.)	VFD (mg/g d.m.)	NFD (mg/g d.m.)
C14:0	8.3 ± 0.4	7.9 ± 0.5	7.3 ± 0.3
C16:0	11.1 ± 0.2	10.9 ± 0.1	10.9 ± 0.2
C18:0	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.0
C16:1n7	1.7 ± 0.1	1.6 ± 0.1	1.4 ± 0.1
C18:1n9	1.8 ± 0.1	1.7 ± 0.0	1.6 ± 0.0
C18:1n7	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
C20:1n9	1.1 ± 0.0	0.9 ± 0.1	0.9 ± 0.1
C22:1n11	1.9 ± 0.0	1.6 ± 0.2	1.5 ± 0.1
C18:2n6	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0
C18:3n3	2.2 ± 0.0	2.1 ± 0.0	2.1 ± 0.0
C18:4n3	13.6 ± 0.3	12.9 ± 0.7	12.8 ± 0.6
C20:4n3	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.0
C20:5n3	11.0 ± 0.2	10.9 ± 0.1	11.0 ± 0.2
C22:6n3	15.0 ± 0.4	14.5 ± 0.3	15.0 ± 0.6

The amount of secondary oxidation products was measured for CF dried with the different drying methods (Fig. 2C). NFD showed a higher TBARS value than AFD and VFD. A lipid is considered rancid when its TBARS value is over 1 (Østerlie, 2000). NFD was the only drying method with TBARS that exceeded this value.

DISCUSSION

The significantly lower drying rates for NFD can partly be explained by the stationary state of the bed during drying. A fluidized state would likely result in an increased drying rate for NFD. However, slower drying rates for nitrogen drying were also observed for other products, such as garlic (Rahmann et al., 2009) and might be related to the drying agent. AFD resulted in the fastest drying of CF, as has previously been reported by Bantle et al. (2009).

The lipids are valuable ingredients in CF and must be preserved in the drying process used. The amount of total lipid present in the dried product decreased with increasing drying rate (Fig. 2A) while the content of phospholipids decreased (Fig. 2B) and FFA increased (Fig. 2A). This can be explained by hydrolytic reactions in the drying process. Phospholipases will hydrolyze phospholipids to FFA and lyso-phospholipids. Lysophospholipids are further hydrolyzed by phospholipases and other lipases (Brockerhoff, 1974).

The decrease in phospholipids correlates well with the formation of FFA (Fig. 3.). However, it cannot explain all the formed FFA. In a typical CF phospholipid, about 80 % of the phospholipid is fatty acids. If all the phospholipid in the zero sample (37 ± 6) were hydrolyzed, the theoretical amount of FFA could be about 30 mg/g dry matter. Compared to the measured amount of 47 mg/g d.m. there is an amount corresponding to 1.7 % of dry weight missing. Wax esters can be hydrolyzed to fatty acid and fatty alcohol however they have been shown to be persistent both in CF and also in krill *post mortem* (Overrein, 2010; Saether et al., 1986). Wax esters were not analyzed in this study. The 20 % of the phospholipid that make up the phospholipid backbone (glycerol, phosphate and a nitrogenous group) will give a small total lipid loss but cannot explain the loss found in this study. Overrein (2010) found a high *post mortem* lipolytic activity in CF indicating a high level of active lipases and phospholipases. Also in her study, the loss of phospholipids could not explain the formation of FFA. Rehydrated dried CF has also been shown to have a high lipolytic activity (Bantle et al., 2009). This study seems to indicate that there is some activity in frozen CF.

Drying temperatures of -17°C (VFD), -8°C (NFD) and -6°C (AFD) did not preserve the lipids completely, since free fatty acids were observed in all dried products (Fig. 2A). Enzyme activity during freeze storage is a well established fact. However, they are slowed down with lower temperature. As the water freezes, the other solutes concentrates and can give various effects on enzyme activity in different mediums. An elevated activity during freezing could occur (Damodaran et al., 2008). This indicates that a temperature of -6 ºC could potentially give a higher enzyme activity than 0 ºC. This has not been tested in CF to our knowledge. Lovern and Olley (1962) showed the highest free fatty acid accumulation at -4 ºC in cod flesh.

In VFD no drying agent is present to react with the product and the drying temperature (-17°C) is lower than in AFD and NFD. It was therefore expected that the amount of FFA should be lowest and the lipids preserved best with VFD. Also NFD (inert drying agent and low temperature) should preserve the lipids better than AFD. However AFD showed the lowest amount of FFA after drying (Fig. 2A) despite the high drying temperature (-6°C) and the presence of a reactive drying agent (air). NFD gave the highest FFA content of 32 % of total lipid. The formation of FFA correlates well with drying rate (Fig. 2A) and not with drying temperature and/or drying method. In NFD, the enzymes were active over a longer period and at a higher temperature resulting in more FFA and lower amount of phospholipids. Even though nitrogen is an inert drying agent some oxidative reactions take place during freeze drying according to the TBARS value. These reactions are influenced by factors such as pro-oxidants and light.

The CF was ground before freeze drying. The lipolytic enzymes are then liberated from the tissue and can potentially give a better enzyme-substrate contact and hence more degradation. Saether et al. (1986) showed a higher protein and phospholipid degradation during storage of homogenized krill, compared to whole krill (*M. norvegica*). However, they found no higher degradation in homogenized samples of *Thysanoessa* species. During grinding in this study, a small rise in temperature could also lead to higher enzyme activity and hence some degradation before the drying started.

The omega-3 fatty acids were preserved equally well in the different freeze drying processes and did not seem to be influenced by the drying rate and drying temperature. Fatty acids released from phospholipids by enzymatic activity were found as FFA. However, FFAs are more easily oxidized and the high content of PUFA gives an even higher oxidation potential (Frankel, 1998).

TBARS values in the different drying methods showed no difference between AFD and VFD, even though air is present in AFD. These samples were not rancid, according to the level for rancidity set by Østerlie (2000) at a TBARS value of 1. According to this value, however, the use of NFD resulted in a rancid product. This could be due to the high amount of FFA. However, if the PUFA in the FFA was oxidized, we would expect a lower value of PUFA in NFD.

The high enzyme activity even in frozen condition suggests that CF needs to be stored in deep frozen state or with its valuable lipids separated out. When drying CF, it is necessary to dry the product as rapidly as possible and to store the dried product in a frozen state. To avoid degradation of lipids by enzymes during the drying processes, it is possible to extract the CF lipids directly on board a fishing vessel equipped with a belt press and a decanter. This would also reduce the need for freeze storage capacity, since the process also dewaters the product at the same time. A drying process for the remaining dry matter on shore could then focus on preservation of the proteins. Since industrial protein preservation normally requires heat treatment, CF could then be dried using hot air, which would also accelerate the drying process.

CONCLUSIONS

AFD and VFD are the most suitable preservation methods for phospholipids (PE and PC) and omega-3 long chain fatty acids such as DHA, EPA and SDA in this study. The low drying rate in NFD resulted in a higher amount of free fatty acids and a reduced amount of phospholipids. CF dried by NFD also showed the highest lipid oxidation.

NOMENCLATURE

AFD	Atmospheric Freeze Drying	-
CF	*Calanus finmarchicus*	-
DHA	Docosahexaenoic acid	-
d.m.	dry matter	-
EPA	Eicosapentaenoic acid	-
FFA	Free fatty acids	-
NFD	Nitrogen Freeze Drying	-
PC	Phosphatidyl choline	-
PE	Phosphatidyl etanolamine	-
SDA	Stearidonic acid	-
TBARS	Thiobarbituric acid reactive compounds	-
VFD	Vacuum Freeze Drying	-
w.w.	wet weight	-

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1993	Kåre Haugan	Dr. scient Bothany	Mutations in the replication control gene trfA of the broad host-range plasmid RK2
1994	Peder Fiske	Dr. scient. Zoology	Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek
1994	Kjell Inge Reitan	Dr. scient Botany	Nutritional effects of algae in first-feeding of marine fish larvae
1994	Nils Røv	Dr. scient Zoology	Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant *Phalacrocorax carbo carbo*
1994	Annette-Susanne Hoepfner	Dr. scient Botany	Tissue culture techniques in propagation and breeding of Red Raspberry (*Rubus idaeus* L.)
1994	Inga Elise Bruteig	Dr. scient Bothany	Distribution, ecology and biomonitoring studies of epiphytic lichens on conifers
1994	Geir Johnsen	Dr. scient Botany	Light harvesting and utilization in marine phytoplankton: Species-specific and photoadaptive responses
1994	Morten Bakken	Dr. scient Zoology	Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, *Vulpes vulpes*

1994	Arne Moksnes	Dr. philos Zoology	Host adaptations towards brood parasitism by the Cockoo
1994	Solveig Bakken	Dr. scient Bothany	Growth and nitrogen status in the moss *Dicranum majus* Sm. as influenced by nitrogen supply
1994	Torbjørn Forseth	Dr. scient Zoology	Bioenergetics in ecological and life history studies of fishes.
1995	Olav Vadstein	Dr. philos Botany	The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions
1995	Hanne Christensen	Dr. scient Zoology	Determinants of Otter *Lutra lutra* distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink *Mustela vision*
1995	Svein Håkon Lorentsen	Dr. scient Zoology	Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition
1995	Chris Jørgen Jensen	Dr. scient Zoology	The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
1995	Martha Kold Bakkevig	Dr. scient Zoology	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport
1995	Vidar Moen	Dr. scient Zoology	Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations
1995	Hans Haavardsholm Blom	Dr. philos Bothany	A revision of the *Schistidium apocarpum* complex in Norway and Sweden
1996	Jorun Skjærmo	Dr. scient Botany	Microbial ecology of early stages of cultivated marine fish; inpact fish-bacterial interactions on growth and survival of larvae
1996	Ola Ugedal	Dr. scient Zoology	Radiocesium turnover in freshwater fishes
1996	Ingibjørg Einarsdottir	Dr. scient Zoology	Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines
1996	Christina M. S. Pereira	Dr. scient Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation
1996	Jan Fredrik Børseth	Dr. scient Zoology	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics
1996	Gunnar Henriksen	Dr. scient Zoology	Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region
1997	Gunvor Øie	Dr. scient Bothany	Eevalution of rotifer *Brachionus plicatilis* quality in early first feeding of turbot *Scophtalmus maximus* L. larvae
1997	Håkon Holien	Dr. scient Botany	Studies of lichens in spurce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters
1997	Ole Reitan	Dr. scient. Zoology	Responses of birds to habitat disturbance due to damming
1997	Jon Arne Grøttum	Dr. scient. Zoology	Physiological effects of reduced water quality on fish in aquaculture
1997	Per Gustav Thingstad	Dr. scient. Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher
1997	Torgeir Nygård	Dr. scient Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as Biomonitors

1997 Signe Nybø	Dr. scient. Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway
1997 Atle Wibe	Dr. scient. Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry
1997 Rolv Lundheim	Dr. scient Zoology	Adaptive and incidental biological ice nucleators
1997 Arild Magne Landa	Dr. scient Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation
1997 Kåre Magne Nielsen	Dr. scient Botany	An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*
1997 Jarle Tufto	Dr. scient Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997 Trygve Hesthagen	Dr. philos Zoology	Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
1997 Trygve Sigholt	Dr. philos Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997 Jan Østnes	Dr. scient Zoology	Cold sensation in adult and neonate birds
1998 Seethaledsumy Visvalingam	Dr. scient Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins
1998 Thor Harald Ringsby	Dr. scient Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998 Erling Johan Solberg	Dr. scient. Zoology	Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment
1998 Sigurd Mjøen Saastad	Dr. scient Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity
1998 Bjarte Mortensen	Dr. scient Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro
1998 Gunnar Austrheim	Dr. scient Botany	Plant biodiversity and land use in subalpine grasslands. – A conservtaion biological approach
1998 Bente Gunnveig Berg	Dr. scient Zoology	Encoding of pheromone information in two related moth species
1999 Kristian Overskaug	Dr. scient Zoology	Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach
1999 Hans Kristen Stenøien	Dr. scient Bothany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)
1999 Trond Arnesen	Dr. scient Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway
1999 Ingvar Stenberg	Dr. scient Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*
1999 Stein Olle Johansen	Dr. scient Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis

1999	Trina Falck Galloway	Dr. scient Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Marianne Giæver	Dr. scient Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic
1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*
1999	Ingrid Bysveen Mjølnerød	Dr. scient Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient Zoology	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*)
1999	Gunnbjørn Bremset	Dr. scient Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999	Sonja Andersen	Dr. scient Bothany	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)
2000	Odd A. Gulseth	Dr. philos Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000	Pål A. Olsvik	Dr. scient Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway
2000	Sigurd Einum	Dr. scient Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001	Jan Ove Evjemo	Dr. scient Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species
2001	Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems

2001	Ingebrigt Uglem	Dr. scient Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001	Bård Gunnar Stokke	Dr. scient Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002	Ronny Aanes	Dr. scient	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002	Mariann Sandsund	Dr. scient Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002	Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002	Frank Rosell	Dr. scient Zoology	The function of scent marking in beaver (*Castor fiber*)
2002	Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002	Terje Thun	Dr.philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002	Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002	Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002	Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and the Ral GTPase from *Drosophila melanogaster*
2002	Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003	Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003	Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003	Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003	Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003	Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003	Marit Stranden	Dr.scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003	Kristian Hassel	Dr.scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003	David Alexander Rae	Dr.scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr.scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003	Eldar Åsgard Bendiksen	Dr.scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt
2004	Torkild Bakken	Dr.scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr.scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar

2004	Tore Brembu	Dr.scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr.scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr.scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*)
2004	Lene Østby	Dr.scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr.scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr.scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr.scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr.scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr.scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr.scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	ph.d Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr.scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr.scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations
2005	Christian Westad	Dr.scient Biology	Motor control of the upper trapezius
2005	Lasse Mork Olsen	ph.d Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	ph.d Biology	Implications of mate choice for the management of small populations
2005	Ariaya Hymete Sahle Dingle	ph.d Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Anders Gravbrøt Finstad	ph.d Biology	Salmonid fishes in a changing climate: The winter challenge
2005	Shimane Washington Makabu	ph.d Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr.scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation

2006	Kari Mette Murvoll	ph.d Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006	Ivar Herfindal	Dr.scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006	Nils Egil Tokle	ph.d Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006	Jan Ove Gjershaug	Dr.philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006	Jon Kristian Skei	Dr.scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006	Johanna Järnegren	ph.d Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity
2006	Bjørn Henrik Hansen	ph.d Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006	Vidar Grøtan	ph.d Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006	Jafari R Kideghesho	ph.d Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006	Anna Maria Billing	ph.d Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006	Henrik Pärn	ph.d Biology	Female ornaments and reproductive biology in the bluethroat
2006	Anders J. Fjellheim	ph.d Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006	P. Andreas Svensson	ph.d Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007	Sindre A. Pedersen	ph.d Biology	Metal binding proteins and antifreeze proteins in the beetle *Tenebrio molitor* - a study on possible competition for the semi-essential amino acid cysteine
2007	Kasper Hancke	ph.d Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007	Tomas Holmern	ph.d Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation
2007	Kari Jørgensen	ph.d Biology	Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth *Heliothis virescens*
2007	Stig Ulland	ph.d Biology	Functional Characterisation of Olfactory Receptor Neurons in the Cabbage Moth, (*Mamestra brassicae* L.) (Lepidoptera, Noctuidae). Gas Chromatography Linked to Single Cell Recordings and Mass Spectrometry
2007	Snorre Henriksen	ph.d Biology	Spatial and temporal variation in herbivore resources at northern latitudes
2007	Roelof Frans May	ph.d Biology	Spatial Ecology of Wolverines in Scandinavia
2007	Vedasto Gabriel Ndibalema	ph.d Biology	Demographic variation, distribution and habitat use between wildebeest sub-populations in the Serengeti National Park, Tanzania

2007	Julius William Nyahongo	ph.d Biology	Depredation of Livestock by wild Carnivores and Illegal Utilization of Natural Resources by Humans in the Western Serengeti, Tanzania
2007	Shombe Ntaraluka Hassan	ph.d Biology	Effects of fire on large herbivores and their forage resources in Serengeti, Tanzania
2007	Per-Arvid Wold	ph.d Biology	Functional development and response to dietary treatment in larval Atlantic cod *(Gadus morhua* L.) Focus on formulated diets and early weaning
2007	Anne Skjetne Mortensen	ph.d Biology	Toxicogenomics of Aryl Hydrocarbon- and Estrogen Receptor Interactions in Fish: Mechanisms and Profiling of Gene Expression Patterns in Chemical Mixture Exposure Scenarios
2008	Brage Bremset Hansen	ph.d Biology	The Svalbard reindeer (*Rangifer tarandus platyrhynchus*) and its food base: plant-herbivore interactions in a high-arctic ecosystem
2008	Jiska van Dijk	ph.d Biology	Wolverine foraging strategies in a multiple-use landscape
2008	Flora John Magige	ph.d Biology	The ecology and behaviour of the Masai Ostrich (Struthio camelus massaicus) in the Serengeti Ecosystem, Tanzania
2008	Bernt Rønning	ph.d Biology	Sources of inter- and intra-individual variation in basal metabolic rate in the zebra finch, (*Taeniopygia guttata*)
2008	Sølvi Wehn	ph.d Biology	Biodiversity dynamics in semi-natural mountain landscapes. - A study of consequences of changed agricultural practices in Eastern Jotunheimen
2008	Trond Moxness Kortner	ph.d Biology	"The Role of Androgens on previtellogenic oocyte growth in Atlantic cod (*Gadus morhua*): Identification and patterns of differentially expressed genes in relation to Stereological Evaluations"
2008	Katarina Mariann Jørgensen	Dr.Scient Biology	The role of platelet activating factor in activation of growth arrested keratinocytes and re-epithelialisation
2008	Tommy Jørstad	ph.d Biology	Statistical Modelling of Gene Expression Data
2008	Anna Kusnierczyk	ph.d Bilogy	*Arabidopsis thaliana* Responses to Aphid Infestation
2008	Jussi Evertsen	ph.d Biology	Herbivore sacoglossans with photosynthetic chloroplasts
2008	John Eilif Hermansen	ph.d Biology	Mediating ecological interests between locals and globals by means of indicators. A study attributed to the asymmetry between stakeholders of tropical forest at Mt. Kilimanjaro, Tanzania
2008	Ragnhild Lyngved	ph.d Biology	Somatic embryogenesis in *Cyclamen persicum.* Biological investigations and educational aspects of cloning
2008	Line Elisabeth Sundt-Hansen	ph.d Biology	Cost of rapid growth in salmonid fishes
2008	Line Johansen	ph.d Biology	Exploring factors underlying fluctuations in white clover populations – clonal growth, population structure and spatial distribution
2009	Astrid Jullumstrø Feuerherm	ph.d Biology	Elucidation of molecular mechanisms for pro-inflammatory phospholipase A2 in chronic disease

2009	Pål Kvello	ph.d Biology	Neurons forming the network involved in gustatory coding and learning in the moth *Heliothis virescens:* Physiological and morphological characterisation, and integration into a standard brain atlas
2009	Trygve Devold Kjellsen	ph.d Biology	Extreme Frost Tolerance in Boreal Conifers
2009	Johan Reinert Vikan	ph.d Biology	Coevolutionary interactions between common cuckoos *Cuculus canorus* and *Fringilla* finches
2009	Zsolt Volent	ph.d Biology	Remote sensing of marine environment: Applied surveillance with focus on optical properties of phytoplankton, coloured organic matter and suspended matter
2009	Lester Rocha	ph.d Biology	Functional responses of perennial grasses to simulated grazing and resource availability
2009	Dennis Ikanda	ph.d Biology	Dimensions of a Human-lion conflict: Ecology of human predation and persecution of African lions (*Panthera leo*) in Tanzania
2010	Huy Quang Nguyen	ph.d Biology	Egg characteristics and development of larval digestive function of cobia (*Rachycentron canadum*) in response to dietary treatments -Focus on formulated diets
2010	Eli Kvingedal	ph.d Biology	Intraspecific competition in stream salmonids: the impact of environment and phenotype
2010	Sverre Lundemo	ph.d Biology	Molecular studies of genetic structuring and demography in *Arabidopsis* from Northern Europe
2010	Iddi Mihijai Mfunda	ph.d Biology	Wildlife Conservation and People's livelihoods: Lessons Learnt and Considerations for Improvements. Tha Case of Serengeti Ecosystem, Tanzania
2010	Anton Tinchov Antonov	ph.d Biology	Why do cuckoos lay strong-shelled eggs? Tests of the puncture resistance hypothesis
2010	Anders Lyngstad	ph.d Biology	Population Ecology of *Eriophorum latifolium*, a Clonal Species in Rich Fen Vegetation
2010	Hilde Færevik	ph.d Biology	Impact of protective clothing on thermal and cognitive responses
2010	Ingerid Brænne Arbo	ph.d Medical technology	Nutritional lifestyle changes – effects of dietary carbohydrate restriction in healthy obese and overweight humans
2010	Yngvild Vindenes	ph.d Biology	Stochastic modeling of finite populations with individual heterogeneity in vital parameters
2010	Hans-Richard Brattbakk	ph.d Medical technology	The effect of macronutrient composition, insulin stimulation, and genetic variation on leukocyte gene expression and possible health benefits
2011	Geir Hysing Bolstad	ph.d Biology	Evolution of Signals: Genetic Architecture, Natural Selection and Adaptive Accuracy
2011	Karen de Jong	ph.d Biology	Operational sex ratio and reproductive behaviour in the two-spotted goby (*Gobiusculus flavescens*)
2011	Ann-Iren Kittang	ph.d Biology	*Arabidopsis thaliana* L. adaptation mechanisms to microgravity through the EMCS MULTIGEN-2 experiment on the ISS:– The science of space experiment integration and adaptation to simulated microgravity
2011	Aline Magdalena Lee	ph.d Biology	Stochastic modeling of mating systems and their effect on population dynamics and genetics
2011	Christopher Gravningen Sørmo	ph.d Biology	Rho GTPases in Plants: Structural analysis of ROP GTPases; genetic and functional studies of MIRO GTPases in *Arabidopsis thaliana*

2011	Grethe Robertsen	ph.d Biology	Relative performance of salmonid phenotypes across environments and competitive intensities
2011	Line-Kristin Larsen	ph.d Biology	Life-history trait dynamics in experimental populations of guppy (*Poecilia reticulata*): the role of breeding regime and captive environment
2011	Maxim A. K. Teichert	ph.d Biology	Regulation in Atlantic salmon (*Salmo salar*): The interaction between habitat and density
2011	Torunn Beate Hancke	ph.d Biology	Use of Pulse Amplitude Modulated (PAM) Fluorescence and Bio-optics for Assessing Microalgal Photosynthesis and Physiology
2011	Sajeda Begum	ph.d Biology	Brood Parasitism in Asian Cuckoos: Different Aspects of Interactions between Cuckoos and their Hosts in Bangladesh
2011	Kari J. K. Attramadal	ph.d Biology	Water treatment as an approach to increase microbial control in the culture of cold water marine larvae
2011	Camilla Kalvatn Egset	ph.d Biology	The Evolvability of Static Allometry: A Case Study
2011	AHM Raihan Sarker	ph.d Biology	Conflict over the conservation of the Asian elephant (*Elephas maximus*) in Bangladesh
2011	Gro Dehli Villanger	ph.d Biology	Effects of complex organohalogen contaminant mixtures on thyroid hormone homeostasis in selected arctic marine mammals
2011	Kari Bjørneraas	ph.d Biology	Spatiotemporal variation in resource utilisation by a large herbivore, the moose
2011	John Odden	ph.d Biology	The ecology of a conflict: Eurasian lynx depredation on domestic sheep
2011	Simen Pedersen	ph.d Biology	Effects of native and introduced cervids on small mammals and birds
2011	Mohsen Falahati-Anbaran	ph.d Biology	Evolutionary consequences of seed banks and seed dispersal in *Arabidopsis*
2012	Jakob Hønborg Hansen	ph.d Biology	Shift work in the offshore vessel fleet: circadian rhythms and cognitive performance
2012	Elin Noreen	ph.d Biology	Consequences of diet quality and age on life-history traits in a small passerine bird
2012	Irja Ida Ratikainen	ph.d Biology	Theoretical and empirical approaches to studying foraging decisions: the past and future of behavioural ecology
2012	Aleksander Handå	ph.d Biology	Cultivation of mussels (*Mytilus edulis):*Feed requirements, storage and integration with salmon (*Salmo salar*) farming
2012	Morten Kraabøl	ph.d Biology	Reproductive and migratory challenges inflicted on migrant brown trour (*Salmo trutta* L) in a heavily modified river
2012	Jisca Huisman	ph.d Biology	Gene flow and natural selection in Atlantic salmon