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Proximate biochemical composition and caloric content calculated from elemental CHN analysis: a stoichiometric concept

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Summary. Carbohydrate, lipid, and protein compositions are stoichiometrically related to organic CHN (carbon, hydrogen, nitrogen) contents. Elemental CHN analyses of total biomass and ash, therefore, provide a basis for the calculation of proximate biochemical composition and bomb caloric value. The classical nitrogen to protein conversion factor (6.25) should be replaced by 5.8 ± 0.13. A linear relation exists between the mass fraction of non-protein carbon and the carbohydrate and lipid content. Residual water in dry organic matter can be estimated with the additional information derived from hydrogen measurements.

The stoichiometric CHN method and direct biochemical analysis agreed within 10% of ash-free dry biomass (for muscle, liver and fat tissue of silver carp; gut contents composed of detritus and algae; commercial fish food). The detrital material, however, had to be corrected for non-protein nitrogen.

A linear relationship between bomb caloric value and organic carbon fractions was derived on the basis of thermodynamic and stoichiometric principles, in agreement with experimental data published for bacteria, algae, protozoa and invertebrates. The highly automatic stoichiometric CHN method for the separation of nutrient contents in biomass extends existing ecophysiological concepts for the construction of balanced carbon and nitrogen, as well as biochemical and energy budgets.

Introduction

Many fundamental methods in ecological and physiological energetics are based on general relationships between elemental, biochemical, and caloric changes in organisms and ecosystems. These relations provide the key to indirect calorimetry (conversion of oxygen consumption into heat dissipation), to the estimation of protein metabolism (from nitrogen excretion), and to the calculation of caloric equivalents of biomass (from organic carbon).

Carbon to energy conversion factors have been published for some bacteria, plant and animal groups (Parsons et al. 1961; Platt and Irwing 1973; Salonen et al. 1976; Finlay and Uhlig 1981). These experimental data prompted us to clarify the thermodynamic and stoichiometric principles which determine the close relationship between organic carbon and caloric content. This required a reinvestigation of stoichiometric relations such as the carbon to nitrogen ratio and the nitrogen content in proteins. More importantly, we developed and tested a method for the separation of carbohydrate, lipid and protein based on ash weight and the CHN (carbon, hydrogen, nitrogen) content of organic matter.

Various measures of heterotrophic energy and nutrient *flow*, in metabolic transformations and trophic dynamics, are closely related to the energetic *state* as defined by the stoichiometric CHN concept. Such flows are regulated for the maintenance of carbon and nitrogen balance in biological systems. Low N:C ratios indicate a low protein content which may limit the nutritional potential of food more effectively than a low caloric value, at least in detritus (Fenchel and Blackburn 1979; Newell 1979). High lipid *or* high carbohydrate contents are responsible for a low N:C ratio. Therefore this commonly used ecological index is neither related to a particular biochemical composition nor to the total energy content of biomass. However, expressions of *weight specific* carbon (w_C), when stated in addition to the N:C ratio, potentially provide sufficient information about the close relationship between organic carbon and nitrogen, biochemical composition, and caloric value. An equivalent concept, based on carbon and nitrogen changes, is used in indirect calorimetry where metabolic energy and nutrient losses are calculated. The stoichiometric CHN method presented here for the determination of biochemical constituents of biomass has certain advantages over traditional biochemical analyses: 1) It is consistent with established ecophysiological concepts for biochemical and energy budget calculations; 2) It requires only small amounts of material (ca. 1 mg dry weight); and 3) Automatic analyzers are available which perform simultaneous CHN measurements in a very short time.

Theory of the stoichiometric CHN concept

The respiratory quotient, RQ (mol CO_2 liberated per mol O_2 consumed) is a well established concept for the calculation of carbohydrate and lipid fractions that are being oxidized (Brody 1945; Kleiber 1961; Blaxter

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Table 1. Elemental CHN composition and bomb caloric values of standard carbohydrate, lipid and protein. C_i is the mass fraction of carbon in carbohydrate (C_K), lipid (C_L) and protein (C_P); H_i is the mass fraction of hydrogen in i; N_i (N_P) is the mass fraction of nitrogen in i (protein). $\Delta_c h_i$ [kJ/(g i)] is the specific enthalpy of combustion for the three substrates i; $\Delta_c h_C$ [kJ/(g C)] is the specific enthalpy of combustion based on the mass of carbon; $\Delta_c H_C$ [kJ/(mol C)] is the molar enthalpy of combustion based on carbon

i	C_i	H_i	N_i	$\Delta_c h_i$	$\Delta_c h_C$	$\Delta_c H_C$
Carbohydrate[a]	0.444	0.062	0.000	−17.5	−39.4	−473
Lipid[b]	0.776	0.114	0.000	−39.5	−50.9	−611
Protein[c]	0.529	0.070	0.173	−23.9	−45.2	−543

[a] Represented by glycogen and starch (Weast 1975)

[b] Atomic composition of plant oil, mussel fat and fat of freshwater and marine fish calculated according to published free fatty acid patterns, bound as triacylglycerol. Phospholipids are not considered separately since phosphoric acid remains in the ash. For the caloric content of lipid see Fig. 3

[c] After Gnaiger (1983a; unpubl.). No correction for sulfur is made although sulfuric acid remains in the ash

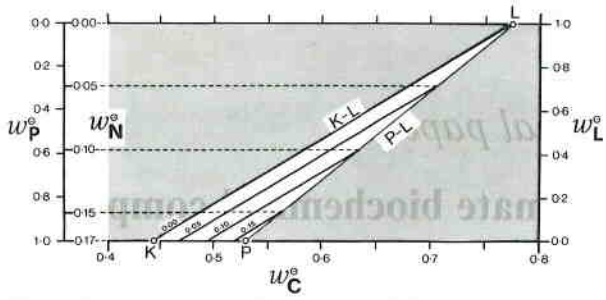

Fig. 1. Mass fraction of protein and lipid, w_P and w_L, as a stoichiometric function of the mass fraction of organic nitrogen and carbon, w_N and w_C. The left ordinate shows the relation of protein and nitrogen for a zero non-protein content (otherwise w_N should be replaced by $x_{PN} \times w_N$; Eq. 3). The K–L line shows the lipid fraction as a function of w_C if only carbohydrate and lipid are present; the P–L line if only protein and lipid are present. This nomogram is based on the pure standard substrates (open circles indicated as K, L, P; Table 1). Therefore, experimental C–N values have to be corrected for residual water (Eq. (4)), and $w_L^\ominus$ values are backcalculated to w_L fractions in real samples by multiplication by $(1 - w_{H_2O})$. Draw a horizontal line from the $w_N^\ominus$ value to the right (e.g. stippled lines) to get the intercept with the P–L line. From this intercept draw a line parallel to the K–L line (e.g. dark sloping lines). Draw a vertical line from $w_C^\ominus$ to get the intercept with the line paralleling K–L. The horizontal projection from this intercept to the right yields the value for the fraction of lipid, $w_L^\ominus$. The mass fraction of carbohydrate is calculated as the difference (Eq. (2)), $w_K = 1 - w_{H_2O} - w_L - w_P$. Combinations of $w_C^\ominus$ and $w_N^\ominus$ extending into the hatched area are inconsistent with the atomic composition of the standard substrates.

Example: for $w_N = 0.094$; $x_{PN} = 1$; $w_C = 0.546$; $w_{H_2O} = 0.06$ we obtain $w_P = 0.547$; $w_N^\ominus = 0.10$; $w_C^\ominus = 0.60$; $w_L^\ominus = 0.32$; $w_L = 0.30$; and $w_K = 0.09$

1967; Gnaiger 1983a). The CHN concept employs stoichiometric calculations similar to those introduced by Voit and Rubner for metabolic studies (see Lusk 1928): The amount of protein and of protein-carbon is calculated from the nitrogen and the carbon to nitrogen ratio in protein. To obtain non-protein carbon, protein-carbon is subtracted from total carbon (excreted as CO_2 or contained in the organics). Similarly the nitrogen quotient, NQ (mol N excreted per mol O_2 consumed), may be used to calculate total non-protein oxygen consumption by subtraction of the oxygen required to oxidize protein from the molar oxygen uptake; with the non-protein RQ derived in this way, it is possible to calculate the relative amounts of carbohydrate and lipid that have been oxidized in addition to the protein. In the stoichiometric CHN method, the ratio corresponding to the RQ is the total organic non-protein carbon [g] per total organic non-protein dry weight [g] (Table 1): When carbohydrate alone is present, this mass ratio is 0.444 (equivalent to $RQ = 1.00$); when lipid is the sole constituent, then the carbon mass fraction is 0.776 (equivalent to $RQ = 0.72$). Non-protein C fractions which are intermediate between 0.444 and 0.776 indicate the mass fractions of carbohydrate and lipid in the mixture according to a linear relationship (Fig. 1, K–L line). The molar N:O_2 ratio (NQ), in turn, can be compared with the N:C mass ratio. For pure protein the latter amounts to 0.327 (equivalent to $NQ = 0.27$; Gnaiger 1983a).

The specific conversion coefficients, the complications arising from the residual water content, and the stoichiometric derivation of the relation between organic carbon and caloric value are discussed below. It is important to note that all CHN and biochemical measurements are expressed as mass fractions (symbol w; unit [g/g]) of the organic (ash-free) dry weight, i.e. the weight (or mass) measurements are divided by the ash-free weight, ${}_{af}W$. In addition, CHN values have to be corrected for the elemental composition of ash to obtain organic mass fractions. For example, the organic carbon fraction in ash-free biomass, w_C [(g organic C)/(g ${}_{af}W$)], is calculated as

$$w_C = \frac{{}_{tot}w_C - {}_{ash}w_C \times w_{ash}}{1 - w_{ash}}, \quad (1)$$

where ${}_{tot}w_C$ is total carbon mass in the total dry biomass [(g total C)/(g ${}_{d}W$)], ${}_{ash}w_C$ is the inorganic carbon fraction in the ash [(g inorganic C)/(g ash)], and w_{ash} is the mass fraction of ash in the dry weight [(g ash)/(g ${}_{d}W$)]. On the basis of organic CHN contents, the total ash-free biomass is separated into the three organic nutrient groups (w_K, w_L and w_P; gram carbohydrate, lipid and protein, respectively, per gram ash-free dry weight) and the residual water fraction (w_{H_2O}; gram residual water per gram ash-free dry weight),

$$1 - w_{H_2O} = w_K + w_L + w_P. \quad (2)$$

1. Protein. Organic nitrogen is traditionally multiplied by 6.25 for conversion into protein, on the basis of a 16% nitrogen content in protein, $N_P = 0.16$ (Lusk 1928; Brody 1945; Winberg 1971). However, the nitrogen fraction in protein as calculated from amino acid compositions of bacteria, algae and aquatic animals (Gnaiger 1983a; unpubl.) was significantly higher than 0.16, N_P averaging 0.173 ± 0.004 S.D. Therefore we propose

Table 2. Stoichiometric parameters for the calculation of proximate biochemical composition (Eqs. (A8)–(A10)), theoretical hydrogen content, and caloric value [kJ/($g_{af}W$)] (Eq. (A16)). The parameters were calculated from the constants given in Table 1, and from the stoichiometric equations derived in the Appendix in the form

$$Y_i = b_{i\ominus} \times (1 - w_{H_2O}) + b_{iC} \times w_C + b_{iN} \times x_{PN} \times w_N$$

Y_i	$b_{i\ominus}$	b_{iC}	b_{iN}
w_K	2.337	−3.012	−4.300
w_L	−1.337	3.012	−1.480
w_P	0.000	0.000	5.780
w_H[a]	−0.0075	0.1566	−0.0307
$\Delta_c h$	11.92	−66.265	−4.436

[a] The hydrogen content of residual water, $0.1006 \times w_{H_2O}$, has to be added to calculate the total ash-free hydrogen fraction (Eq. (A12))

the new nitrogen-protein conversion factor, $P_N = 1/N_P = 5.8$, which can be applied to protein-derived nitrogen in aquatic organisms with an accuracy of 3%.

Non-protein nitrogen may cause a further reduction in this conversion factor, since

$$w_P = P_N \times x_{PN} \times w_N \quad (3)$$

where w_P and w_N are the mass fractions of protein and total organic N respectively, and $x_{PN} \leqq 1$ is the fraction of protein-N per total organic nitrogen in the sample.

2. Carbohydrate and lipid. The stoichiometric equations relating proximate composition to organic CHN are derived in the Appendix. The *b*-parameters are a function of the standard substrates (Table 1) and are summarized in Table 2. Figure 1 is a graphical representation of the stoichiometric CHN concept, and can be used as a nomogram.

3. Residual water. Residual water originates from the contamination of the dry, hygroscopic sample during contact with a humid atmosphere (free water), and is due to water retention during the drying procedure (bound water). Another category of residual water is the chemically bound water that is not accounted for in the standard substrates (Table 1). For instance, due to the binding of water upon hydrolytic cleavage of glycogen, glucose contains 11% chemically bound water relative to glycogen. Correspondingly, free amino acids contain 16% chemically bound water relative to protein. With free amino acids and other monomers amounting to 10–15% of the organic mass, the chemically bound water fraction ranges between 0.01 and 0.02.

Underestimation of w_{H_2O} yields erroneously low organic mass fractions "corrected" to be free of residual water, e.g. for carbon, $w_C^{\ominus}$,

$$w_C^{\ominus} = \frac{w_C}{1 - w_{H_2O}}, \quad (4)$$

and consequently the calculated lipid fraction would be too low (compare Fig. 1 and 2). However, the incorporation of adequately measured hydrogen fractions into the stoichiometric concept provides an estimate of residual water (see Appendix).

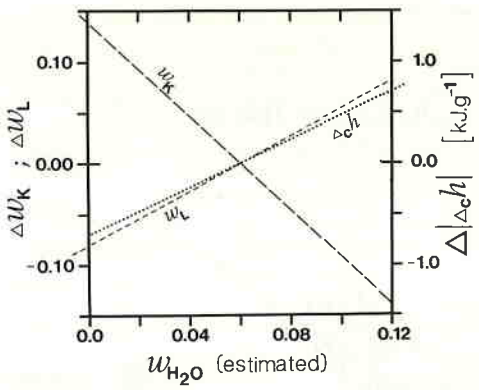

Fig. 2. Effect of the mass fraction of residual water, w_{H_2O}, on the stoichiometric estimates of the mass fractions of carbohydrate and lipid, w_K and w_L, and on the absolute caloric value, $|\Delta_c h|$. For a 1% increase in the estimate of w_{H_2O} ($\Delta w_{H_2O} = 0.01$), w_K decreases by 2.3%, w_L increases by 1.3%, and $|\Delta_c h|$ increases ($\Delta_c h$ becomes more negative) by 0.12 kJ/($g_{af}W$)

4. Bomb caloric value. The specific enthalpy of combustion of organic matter, $\Delta_c h$ [kJ/($g_{af}W$)], is the sum of the enthalpies of combustion of the biochemical components (Table 1) in proportion to their respective mass fractions,

$$\Delta_c h = \Delta_c h_K \times w_K + \Delta_c h_L \times w_L + \Delta_c h_P \times w_P. \quad (5)$$

The nitrogen content exerts a small influence on the specific caloric values of biological samples. The stoichiometric b_{hN} parameter (for $\Delta_c h$) is only 6% of b_{hC} (Table 2); moreover, w_N is typically below 10 to 30% of w_C (N:C ratio between 0.1 and 0.3). Consequently the nitrogen fraction explains theoretically just 1% of the bomb calorimetric variability, and this is within experimental noise. Hence w_N can be substituted by an average nitrogen fraction of 0.1 in the equation for $\Delta_c h$ (Table 2), yielding the linear relation between bomb calorimetric equivalent and mass fraction of organic carbon,

$$\Delta_c h = 11.5 \times (1 - w_{H_2O}) - 66.27 \times w_C. \quad (6)$$

The stoichiometrically derived linear dependence of $\Delta_c h$ on w_C is in accord with a wide range of biochemical substances (Fig. 3).

With increasing estimates of residual water content, the theoretical enthalpy of combustion increases (becoming more negative), despite the diminished organic content, $w^{\ominus} = 1 - w_{H_2O}$. This is due to the high caloric value of lipid, the calculated fraction of which increases with the higher estimate of w_{H_2O}, at the expense of carbohydrate (Fig. 2). Care must be taken to apply Eq. (6) to C–N combinations only if the calculated biochemical fractions are within the stoichiometrically consistent range (Fig. 1). Slightly negative results for carbohydrate or lipid may be due to errors in elemental analysis if one or both of the nutrient groups are actually near zero (e.g. in muscle or fat tissue). In this case negative values should be set to zero and others

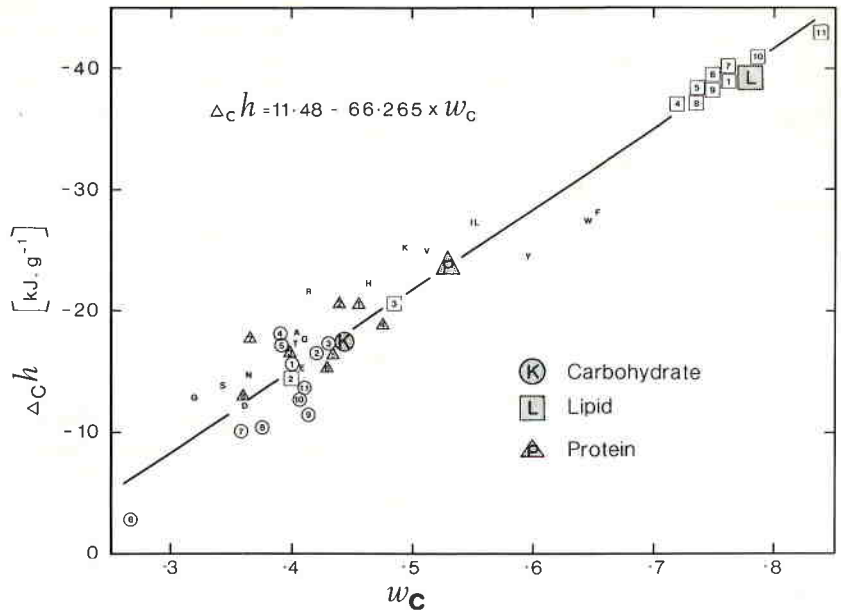

Fig. 3. Specific enthalpy of combustion, $\Delta_c h$, as a function of the mass fraction of carbon, w_C, in biochemical substances. K, L and P are defined in Table 1. The *straight line* was calculated according to stoichiometric relations with the nitrogen fraction taken as 0.10 and for $w_{H_2O}=0$ (Eq. (6)). The values for other organic compounds were selected from Domalski (1972). Numbers in *circles:* (1) hexoses, pentoses; (2) disaccharides; (3) oligosaccharides; (4) glycerol, (5) arabitol, mannitol; (6) oxalic acid; (7) malic acid; (8) citric acid; (9) fumaric acid; (10) succinic acid; (11) ascorbic acid. Numbers in *squares:* [1] average free fatty acid in standard triacylglycerol, and oleic acid; [2] acetic acid; [3] propionic acid; [4] lauric acid; [5] myristic acid; [6] palmitic acid; [7] stearic acid; [8] trilaurateglycerol; [9] trimyristateglycerol; [10] trierucateglycerol; [11] cholesterol. Numbers in *triangles:* ⚠ average free amino acid of standard protein; ⚠ adenine; ⚠ guanine; ⚠ thymine; ⚠ cytosine; ⚠ uracil; ⚠ creatine; ⚠ uric acid; small *letters:* free amino acids

corrected to obey Eq. (2). $\Delta_c h$ can then be recalculated according to Eq. (5).

Materials and methods

For experimental tests of the stoichiometric theory, Chinese silver carp, *Hypophtalmichthys molitrix* (Tolstolop; 2+; 200–500 g wet weight) were collected from fish ponds in Szarvas, Hungary. White muscle from the dorsal region, liver, fat, and total gut contents were pooled from 5–10 healthy individuals. Some analyses were made of carp food (Tagger, Austria) and of faeces of *Rutilus rutilus* fed on this diet. After storage in the deep-freeze, subsamples were dried for 80 h to constant weight at 60 or 105° C, or by freeze drying. The dry material was homogenized in a vibrating powder mill and stored in a desiccator over silicagel. Inorganic fractions, w_{ash}, were determined after ashing at 450° C for 12 h.

1. CHN analysis

Triplicate samples of 1–4 mg $_dW$ were sealed in tin boats and weighed on a Cahn 25 electro microbalance. Samples were either stored in a desiccator or immediately transferred to the automatic sampler of a Carlo Erba (1160) Elemental Analyzer. The combustion temperature was 1,025° C. Inorganic fractions were determined in the ash for corrections according to Eq. (1). Cyclohexanone-2,4-dinitrophenylhydrazone served as the reference. The automatic sampler contained up to 50 samples and was closed by an air-tight perspex box with silicagel to keep the samples dry until completion of the analyses (up to 9 h). In separate experiments the weights of different samples were measured at intervals with and without the perspex cover, to control for water uptake or loss from or to the atmosphere. For future studies we recommend redrying of the samples in the oven after sealing them into the tin boats, prior to weight and CHN analyses.

2. Biochemical analysis

Freeze-dried samples were analyzed in triplicate by standard biochemical methods for ecological materials (Dowgiallo 1975). NaOH soluble protein was measured with the Folin-Ciocalteau reagent (modified method after Lowry et al. 1951) against a bovine albumin standard (Sigma No A-4503). The water content in the standard of 3% was corrected for. Starch type and structural carbohydrates were hydrolyzed in 1 mol/dm^3 and in 79% sulfuric acid, respectively. The colorimetric determination with the phenol reagent was a modification after Dubois et al. (1956). Glucose was used as a reference in the colour reaction. The resulting weight fractions were multiplied by 0.90 to express carbohydrate in terms of glycogen equivalents. Carbohydrate fractions of gut contents, food and faeces were not only measured in triplicate but also on several days. Lipids were determined gravimetrically. An extraction in warm chloroform-methanol (2:1) solvent, to dissociate lipid-protein complexes, was followed by a re-extraction in a chloroform-petroleum ether (1:1) mixture.

Results

1. Residual water and hydrogen contents

Water uptake by the sample has to be strictly avoided before dry weight determination and combustion in CHN analysis, to obtain reliable hydrogen measurements. Dry tissue encapsulated in CHN tin boats absorbed water rapidly, when no further precautions were taken to isolate the samples from the humid atmosphere (Fig. 4). Water uptake depended on the quality of the sample, and was insiginificant in fat tissue and in the ash of muscle.

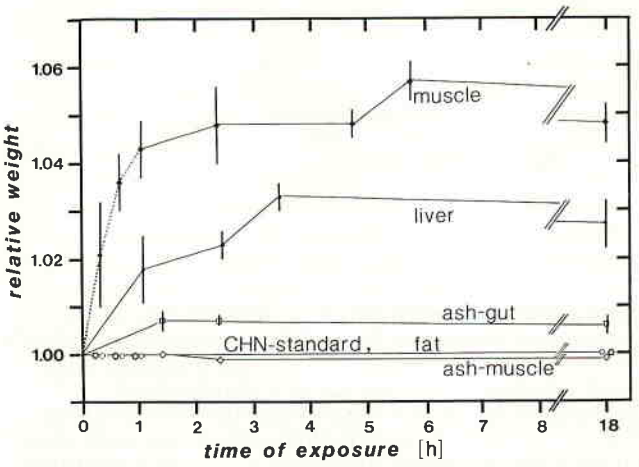

Fig. 4. Gravimetric determination of water uptake of oven dried tissue (muscle, liver, fat; 60° C), ash (of muscle and gut contents), and of the CHN standard (cyclohexanone-2,4-dinitrophenylhydrazone) after encapsulation in tin CHN sample boats. *Full lines:* sample boats in the automatic sampler on the CHN analyzer; *dotted line:* sample boat in the room; *bars:* S.D. of the mean of 3 replicates. Individual samples ranged from 1 to 5 mg, excluding the tin boats

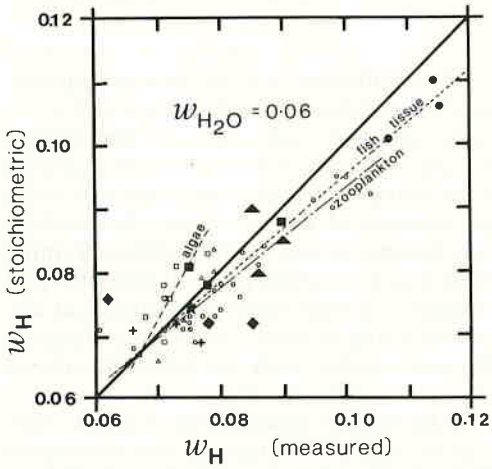

Fig. 5. Mass fraction of hydrogen, w_H, stoichiometrically derived from organic C and N fractions (stoichiometric) versus measured hydrogen fractions in fish tissues (*dark symbols; r* = 0.926) and in various organisms (*open symbols*). The stoichiometric calculation incorporated a residual water fraction of 0.06 (Table 2). ◆ muscle; ▲ liver, ● fat; ■ gut contents; + carp food and faeces; ★ *Mytilus edulis* (unpubl.); □ algae (data from Parsons et al. 1961; $r = 0.800$); ○ marine zooplankton (stoichiometrically consistent data from Omori 1969; $r = 0.933$); △ freshwater zooplankton (data from Baudouin and Ravera 1972). *Full line:* ideal correspondence; *other lines:* calculated regressions using Bartlett's method of best fit

Table 3. Mass fraction of ash, carbon, hydrogen and nitrogen in freeze-dried muscle, liver, fat, and gut contents of silver carp (*Hypophtalmichthys molitrix*). tot – fractions based on total dry weight; af – fractions based on ash-free dry weight and corrected for the CHN contents in ash (e.g. Eq. 1); ash – CHN fractions in ash based on ash weight; n.d. – not detected

		w_{ash}	w_C	w_H	w_N
Muscle	tot	0.079	0.461	0.057	0.142
	af		0.500	0.062	0.154
	ash		0.002	0.000	0.004
Liver	tot	0.064	0.511	0.078	0.092
	af		0.536	0.083	0.095
	ash		0.146	0.000	0.045
Fat	tot	0.002	0.707	0.114	0.009
	af		0.708	0.114	0.009
	ash		n.d.	n.d.	n.d.
Gut	tot	0.474	0.310	0.049	0.041
	af		0.582	0.090	0.075
	ash		0.008	0.004	0.003

We kept the automatic CHN sampler under a dry atmosphere for all elemental analyses. Later measurements, however, showed that muscle samples lost weight under these conditions. Some water contamination must have occurred during homogenizing and filling of the material into the tin capsules. This masked the effect of the different drying methods on the stoichiometrically derived residual water fractions (see Appendix). Nevertheless, agreement was obtained between measured and stoichiometrically calculated hydrogen fractions on the basis of a generally estimated residual water fraction of 0.06 (Fig. 5).

2. Organic CHN, stoichiometric and biochemical analyses

The results pertaining to organic and inorganic CHN contents are shown in Table 3. The high ash and low nitrogen level in the gut contents indicate a predominance of detrital particles filtered by silver carp. Carbon and nitrogen in ash did not exceed 1% of the ash-weight except for liver. Results of one pooled sample of the ash of fat tissue were not different from blank determinations. The ash-hydrogen contents were all below the limit of detection, except in the gut material.

The C and N combinations of the freeze-dried samples were stoichiometrically consistent when corrected for a residual water fraction of 0.06. The sums of the biochemically determined fractions of carbohydrate, lipid and protein were close to the expected value of $w^{\ominus} = 0.94$, except for the gut contents (see legend to Fig. 6).

Agreement between stoichiometrically calculated and biochemically determined proximate biochemical composition was better than 10% of the ash-free dry weight for all tissues, fish food and faeces (Fig. 6). In the gut, however, the protein estimation based on organic nitrogen was twice as high as the colorimetric

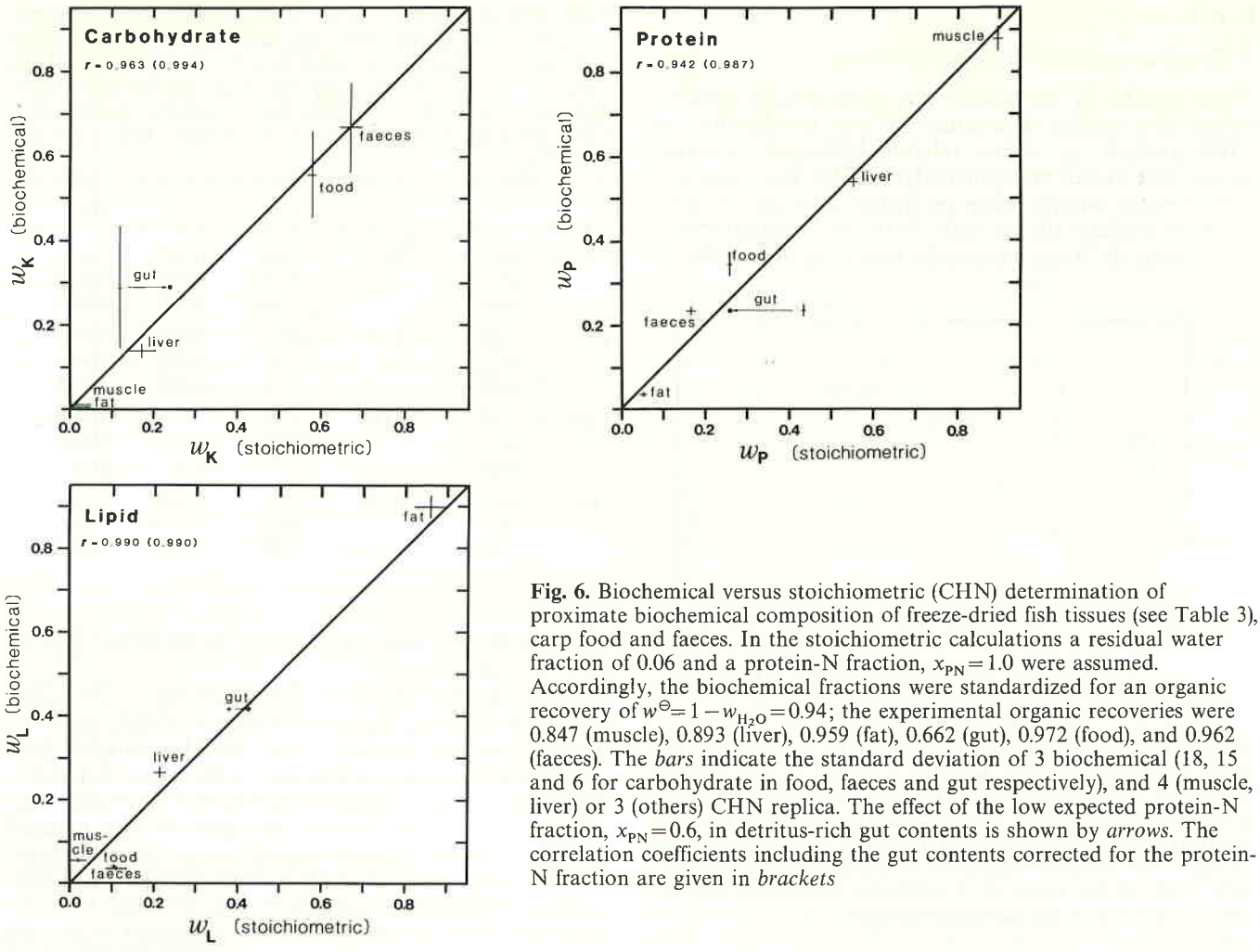

Fig. 6. Biochemical versus stoichiometric (CHN) determination of proximate biochemical composition of freeze-dried fish tissues (see Table 3), carp food and faeces. In the stoichiometric calculations a residual water fraction of 0.06 and a protein-N fraction, $x_{PN}=1.0$ were assumed. Accordingly, the biochemical fractions were standardized for an organic recovery of $w^{\ominus}=1-w_{H_2O}=0.94$; the experimental organic recoveries were 0.847 (muscle), 0.893 (liver), 0.959 (fat), 0.662 (gut), 0.972 (food), and 0.962 (faeces). The *bars* indicate the standard deviation of 3 biochemical (18, 15 and 6 for carbohydrate in food, faeces and gut respectively), and 4 (muscle, liver) or 3 (others) CHN replica. The effect of the low expected protein-N fraction, $x_{PN}=0.6$, in detritus-rich gut contents is shown by *arrows*. The correlation coefficients including the gut contents corrected for the protein-N fraction are given in *brackets*

Table 4. Specific enthalpy of combustion (caloric value) of ash-free dry tissues and gut contents of silver carp, calculated according to the stoichiometric equation (Table 2) for an estimated residual water content of 0.06, irrespective of the drying temperature or freeze drying (f.d.). The N:C mass ratio which is independent of residual water content is shown for comparison. Means and S.D. of 3 replicates (4 in freeze-dried muscle and liver)

	$\Delta_c h$ [kJ/g]			$w_N:w_C$		
	f.d.	60° C	105° C	f.d.	60° C	105° C
Muscle	−22.6	−22.5[a]	−22.5[a]	0.308	0.323	0.309
S.D.	0.48			0.0052	0.0093	0.0015
Liver	−24.7	−26.7	−26.9	0.177	0.159	0.187
S.D.	0.38	0.18	0.62	0.0115	0.0044	0.0100
Fat	−35.8	−32.5	−34.3	0.013	0.026	0.022
S.D.	0.76	1.48	1.19	0.0056	0.0140	0.0142
Gut	−27.7	−23.5	−24.8	0.129	0.131	0.134
S.D.	0.10	0.32	1.59	0.0013	0.0016	0.0019

[a] Corrected for marginally inconsistent values

determination. This indicates a high non-protein nitrogen content of the detritus (40%; Cowey and Corner 1963) which is also consistent with the low biochemical recovery in the gut material. Correction for $x_{PN}=0.6$ (Eq. 3) yielded agreement between the stoichiometric and biochemical measure of protein. Since the absolute value of the b_{KN} parameter (for carbohydrate) is three times higher than b_{LN} (for lipid; Table 2), the effect of non-protein nitrogen is particularly pronounced in the stoichiometric calculation of the carbohydrate fraction. This differential result agrees with the biochemical test (Fig. 6).

The bomb calorimetric equivalents derived stoichiometrically from elemental analyses are shown in Table 4. An effect of the drying techniques exerted on the chemical composition is indicated by the differences in the N:C mass ratios which are not a function of residual water content. The N:C ratio, however, contains insufficient information for stoichiometric analysis (see e.g. Hayashi 1983).

The agreement of stoichiometrically predicted and experimentally determined bomb calorimetric values from various sources was sufficiently convincing (Fig. 7) that we felt no need for conventional bomb calorimetric experiments with our test samples.

Discussion

1. Proximate biochemical analysis

For ecological purposes, body constituents and nutrients should be distinguished on an energetic and metabolic basis rather than in strictly chemical terms. Such categories are necessarily heterogenous, and it may seem difficult to provide standards for carbohydrate, lipid and protein that are generally valid for living and decomposing organic matter. In this respect, stoichiometric analysis provides high flexibility, since chemical background information about specific material can be incorporated in the definition of appropriate standards (Maciolec 1962). For colorimetric methods the problem of the proper choice of a standard is more difficult, since the colorimetric response of a complex organic extract is neither strictly proportional to mass nor to molar quantities (Dubois et al. 1956; Lowry et al. 1951). Moreover, effects of incomplete extraction, coextraction of impurities, and colorimetric interferences are unpredictable and must be considered as being significant, although they may be counteractive, occasionally cancelling out in the result (Blažka 1966; Dowgiallo 1975; Holland and Gabbott 1971; Strickland and Parsons 1960).

In aquatic animals compounds containing non-protein nitrogen range from 2 to 24% of total nitrogen ($x_{PN}=0.74$ to 0.98; excluding elasmobranchs) (Cowey and Corner 1963; Craig et al. 1978; Vijverberg and Frank 1976). Free amino acids, oligopeptides and amino acid derivatives are predominant sources of non-protein nitrogen content. These substances are not distinguished from protein in terms of nutritional requirements and energetic equivalents, and therefore should be incorporated within the "protein" fraction. In particular, nucleic acids are not discerned as a fourth biochemical group in most bioenergetic studies. Consequently, the pyrimidines and purines should be accounted for in the protein, while the ribose moiety should be subsumed in the carbohydrate fraction. However, the protein-related nitrogen fraction (x_{PN}) is typically low in algae and detritus (Conover 1975; Maciolec 1962; Rice 1982; Tenore 1981). Any non-protein nitrogen decreases the newly proposed nitrogen-protein conversion factor of 5.8.

Accurate separations of organic and inorganic carbon and mass (Froelich 1980) are fundamental for the stoichiometric calculation of carbohydrate and lipid fractions. In direct biochemical determinations incomplete or excessive mass recoveries, up to 15–20% of the ash-free dry weight, are not exceptional and indicate the magnitude of the inherent errors. Residual water comprises a recognized (Paine 1971) but frequently underestimated fraction. Beukema and de Bruin (1979) interpreted the 3% weight loss of biomass sequentially dried at 60 and 100° C as residual water, but this is probably an underestimate due to the presence of crystalline water. Finlay and Uhlig (1981) related an abnormally high water retention to the salt content of marine samples. No biological and energetic significance has been ascribed to the hydrogen content of biological matter, whence the results of hydrogen determinations (together with C and N) have frequently not been reported. This omission is unfortunate, since the measured hydrogen fraction contains indispensible information for the stoichiometric calculation of residual water (see Appendix and Fig. 5). For carefully redried tissues of marine bivalves, the residual water fraction calculated according to Eq. (A13) averaged 0.06 ± 0.006 (unpubl.). Due to uncertainties in the present determinations the magnitude of absolute errors in stoichiometric CHN analysis (Fig. 2) is comparable to the inaccuracies of direct biochemical methods.

2. Combustion calorimetry and organic carbon: the stoichiometric concept vs. regression analysis

Regression analysis frequently conceals the lack of a quantitative concept. The calculation of "predictive" numerical regressions hides the merely descriptive nature of the derived quantities though it may satisfy the practical and even some of the academic concern about the respective relationships. Apparently this has been the case in studies on caloric equivalents of biomass. While the basic theory remained to be developed, the connection between carbon content and enthalpy of combustion has been descriptively outlined only (Finlay and Uhlig 1981; Platt et al. 1969; Platt and Irwin 1973; Salonen et al. 1976). These studies point to the high variability of the specific enthalpy of combustion, $\Delta_c h$ [kJ/($g_{af} W$)], as compared to the relatively narrow range of energy equivalents based on carbon, $\Delta_c h_C$ [kJ/(g C)] (see Table 1). Salonen et al. (1976) found a significant correlation between the carbon specific energy and carbon content, but they did not realize that the relationship must be hyperbolic instead of linear. This follows from the simple combination of the relation

$$\Delta_c h_C = \frac{\Delta_c h}{w_C} \tag{7}$$

with Eq. (6):

$$\Delta_c h_C = -66.3 + \frac{11.49 \times (1 - w_{H_2O})}{w_C} \quad [\text{kJ/(g C)}] \tag{8.1}$$

$$\Delta_c H_C = -796 + \frac{137.8 \times (1 - w_{H_2O})}{w_C} \quad [\text{kJ/(mol C)}] \tag{8.2}$$

$\Delta_c H_C$, the molar combustion enthalpy based on carbon, is obtained from the multiplication of Eq. (8.1) by the relative atomic mass of carbon (12.01). The stoichiometric theory on caloric equivalents contains the hidden concept of average bond energies. Simultaneously, however, corrections for the predominant specific effects of molecular structure on the bond energies are incorporated into Eq. (8) (see also Eq. (A16)) by reference to the energetic characteristics of the main substrate groups (Fig. 3).

We calculated the regression for the data shown in Fig. 7 after selecting the 33 samples with stoichiometrically consistent CHN combinations (see Fig. 1 and 5). This experimental relationship ($r = -0.605$),

$$\Delta_c H_C = -788 + \frac{124.5}{w_C},$$

supports the independently derived stoichiometric concept (Eq. (8)) and the approximation for the residual

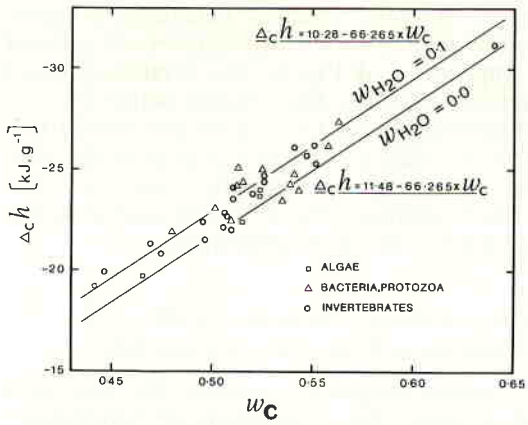

Fig. 7. Specific enthalpy of combustion (energy content) of biomass, $\Delta_c h$, as a function of the mass fraction of organic carbon, w_C (based on ash-free biomass). The two *lines* show the stoichiometrically derived relationship for different residual water fractions (compare Fig. 3). Original data from Finlay and Uhlig (1981), Parsons et al. (1961) and Salonen et al. (1976)

water fraction of 0.06. Our theory predicts that carbon content suffices to determine the caloric content of biomass without significant loss of accuracy due to omission of the nitrogen term (Eq. (6)). Actually, multiple regression analysis of the experimental data did not improve the coefficient of determination (Salonen et al. 1976).

A nitrogen correction for caloric equivalents of biomass was proposed by Kersting (1972). The heat equivalent of ammonia should be subtracted from the combustion values to account for the fact that the catabolic process in animals (catabolic heat equivalent) is different from the combustion process observed in bomb calorimetry (total combustion heat equivalent). Regression of combustion values, after ammonia-nitrogen correction, versus carbon content (Finlay and Uhlig 1981; Salonen et al. 1976), however, implies that the combustion process in the bomb calorimeter may be incomplete. Since this assumption is not supported by experimental findings (Head and Good 1979; Kersting 1972) the nitrogen correction is meaningless in expressing energy equivalents as a function of carbon content.

There are also ecological reasons to dismiss the nitrogen correction. Since the nitrogenous endproduct is different in different organisms (many bacteria oxidize ammonia, which is the principal endproduct in most aquatic animals; mammals and birds produce mainly urea and uric acid repsectively), the N-corrected energy equivalent changes along the food chain for the same amount of protein. This detracts from the benefits of the energy concept which reside in its generality.

The original concept of nitrogen correction was formulated in the context of direct (metabolic) calorimetry where actually measured heat dissipation is compared with the heat changes calculated for specific catabolic reactions (Rubner 1984; for aquatic animals see Gnaiger 1983a; b). In conjunction with indirect calorimetry and with the use of combustion-based energy equivalents of oxygen consumption, consistent energy budgets can be constructed without the complications inferred by the nitrogen correction (Gnaiger 1983a). In agreement with Winberg (1971) we recommend that the nitrogen correction for bomb calorimetric values be abandoned, since combustion calorimetry provides the most general reference in ecological energy budget calculations. The stoichiometric CHN concept can replace conventional bomb calorimetry in most cases, not only because automatic CHN analysis is more practical and caloric values can be accurately calculated (Fig. 7), but also because it provides simultaneous information on proximate biochemical composition.

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Appendix

Derivation of stoichiometric CHN equations

For the purpose of proximate biochemical analysis (Eq. (2)) we define the mass fraction of organic carbon, w_C, as being composed of the carbon in carbohydrate, lipid and protein, C(K), C(L) and C(P) respectively,

$$w_C = C(K) + C(L) + C(P). \quad (A1)$$

For identifying the relative proportions of carbon originating from the three substrate categories, we make use of the substrate specific mass fractions of carbon in K, L and P, namely C_K, C_L and C_P (Table 1). The protein-derived carbon fraction in ash-free biomass is (see Eq. 3)

$$C(P) = C_P \times w_P = (C_P : N_P) \times x_{PN} \times w_N. \quad (A2)$$

The remaining non-protein carbon, $w_C - C(P)$, is contained in carbohydrate and lipid,

$$C(K) = C_K \times w_K \quad (A3)$$

$$C(L) = C_L \times w_L. \quad (A4)$$

We solve now Eq. (A3) in the form of w_K and substitute for C(K) from Eq. (A1),

$$w_K = \frac{w_C - C(L) - C(P)}{C_K}. \quad (A5)$$

Further substituting Eq. (A2) and (A4) into Eq. (A5) yields

$$w_K = \frac{w_C - C_L \times w_L - (C_P : N_P) \times x_{PN} \times w_N}{C_K}. \quad (A6)$$

From Eq. (2) we get

$$w_L = (1 - w_{H_2O}) - w_K - w_P. \quad (A7)$$

By substituting in Eq. (A7) for w_P and w_K from Eq. (3) and Eq. (A6) respectively, the stoichiometric equation

for the lipid fraction is obtained after rearranging as

$$w_L = b_{L\ominus} \times (1 - w_{H_2O}) + b_{LC} \times w_C + b_{LN} \times x_{PN} \times w_N. \quad (A8)$$

where the parameters are

$$b_{L\ominus} = -\frac{C_K}{C_L - C_K} \quad (A8.1)$$

$$b_{LC} = \frac{1}{C_L - C_K} \quad (A8.2)$$

$$b_{LN} = -\frac{C_P - C_K}{N_P \times (C_L - C_K)}. \quad (A8.3)$$

The stoichiometric parameters for the carbohydrate fraction, w_K, are derived in the same way,

$$b_{K\ominus} = \frac{C_L}{C_L - C_K} \quad (A9.1)$$

$$b_{KC} = -\frac{1}{C_L - C_K} \quad (A9.2)$$

$$b_{KN} = -\frac{C_L - C_P}{N_P \times (C_L - C_K)}. \quad (A9.3)$$

For the protein fraction, w_P, the stoichiometric parameters are given directly by Eq. (3); $b_{P\ominus}$ and b_{PC} are zero, and

$$b_{PN} = P_N = \frac{1}{N_P}. \quad (A10)$$

The three substrate fractions, w_i (i.e. w_K, w_L, w_P), determine the organic hydrogen content according to the substrate-specific hydrogen fractions, H_i (i.e. H_K, H_L, H_P; see Table 1). Residual water adds to the total hydrogen fraction in the ash-free biomass, according to the hydrogen mass fraction in water (0.1006),

$$w_H = \sum_i (H_i \times w_i) + 0.1006 \times w_{H_2O}. \quad (A11)$$

If the substrate fractions w_i are calculated stoichiometrically (Table 2), then the theoretical hydrogen fraction is obtained as

$$w_H = \sum_i (H_i \times b_{i\ominus}) \times (1 - w_{H_2O}) + \sum_i (H_i \times b_{iC}) \times w_C + \sum_i (H_i \times b_{iN}) \times x_{PN} \times w_N + 0.1006 \times w_{H_2O}. \quad (A12)$$

Since w_H is automatically measured in CHN analyzers, Eq. (A13) can be used to calculate the residual water fraction,

$$w_{H_2O} = b_{H_2O\ominus} + b_{H_2OC} \times w_C + b_{H_2ON} \times x_{PN} \times w_N + b_{H_2OH} \times w_H \quad (A13)$$

where the parameters are

$$b_{H_2OH} = \frac{1}{0.1006 - b_{K\ominus} \times H_K - b_{L\ominus} \times H_L} \quad (A13.1)$$

$$b_{H_2O\ominus} = -b_{H_2OH} \times 0.1006 + 1 \quad (A13.2)$$

$$b_{H_2OC} = -b_{H_2OH} \times \frac{H_L - H_K}{C_L - C_K} \quad (A13.3)$$

$$b_{H_2ON} = -b_{H_2OH} \times (b_{KN} \times H_K + b_{LN} \times H_L + b_{PN} \times H_P). \quad (A13.4)$$

Inserting the stoichiometric values from Tables 1 and 2 yields

$$w_{H_2O} = 0.0697 - 1.4483 \times w_C + 0.2840 \times x_{PN} \times w_N + 9.2471 \times w_H. \quad (A14)$$

All stochastic errors accumulate in the calculation of w_{H_2O} (Eq. (A13)). Especially if uncertainties exist as to moisture contamination after weight measurements, the comparison of theoretical (Eq. (A12)) and experimental w_H values on the basis of a constant residual water content for a whole experimental series (Fig. 5) may give more reliable results than inserting Eq. (A13) into (A8).

The additive approach of Eq. (A11) and (A12) is also valid for the enthalpy of combustion (compare Eq. (7)),

$$\Delta_c h = \sum_i (\Delta_c h_i \times w_i) \quad (A15)$$

where i again represents K, L and P. Substituting for the w_i from Eq. (A8)–(A10) yields

$$\Delta_c h = \sum_i (\Delta_c h_i \times b_{i\ominus}) \times (1 - w_{H_2O}) + \sum_i (\Delta_c h_i \times b_{iC}) \times w_C + \sum_i (\Delta_c h_i \times b_{iN}) \times x_{PN} \times w_N. \quad (A16)$$

The stoichiometric parameters that are summarized in Table 2 were obtained by inserting the constants for the standard substrates (Table 1) into the relevant equations of this Appendix.

List of symbols: The general symbols used are in accord with IUPAC (1979) recommendations

Symbol Description and Units

$b_{i\ominus}$	stoichiometric parameter for i, relating to the organic dry weight fraction corrected for residual water
b_{iC}	stoichiometric parameter for i, relating to the organic C fraction
b_{iN}	stoichiometric parameter for i, relating to organic nitrogen corrected for the protein-N fraction; $b_{PN} = P_N$
C_i	mass fraction of carbon in substance i (C_K, C_L, C_P)
$C(i)$	$= C_i \times w_i$; mass fraction of carbon originating from substance i in ash-free dry biomass [C(K), C(L), C(P)]
H_i	mass fraction of hydrogen in substance i (H_K, H_L, H_P)
$\Delta_c h$	specific enthalpy of combustion of ash-free dry biomass, caloric value [kJ/($g_{af}W$)]
$\Delta_c h_C$	$= \Delta_c h / w_C$; specific enthalpy of combustion of ash-free dry biomass on a carbon mass basis [kJ/(g C)]
$\Delta_c h_i$	specific enthalpy of combustion of substance i ($\Delta_c h_K$, $\Delta_c h_L$, $\Delta_c h_P$) [kJ/(g i)]
$\Delta_c H_C$	$= \Delta_c h_C \times 12.01$; molar enthalpy of combustion based on carbon [kJ/(mol C)]
K	carbohydrate and carbohydrate equivalent
L	lipid and lipid equivalent
N_P	mass fraction of nitrogen in protein (=0.17)
NQ	nitrogen quotient [(mol N)/(mol O_2)]
P	protein and protein equivalent
P_N	protein-nitrogen mass ratio, i.e. the stoichiometric pa-

rameter, b_{PN}, for nitrogen (corrected for x_{PN}) to protein conversion (=5.8)

RQ respiratory quotient [(mol CO_2)/(mol O_2)]

$w^{\ominus}$ $=1-w_{H_2O}$; mass fraction of ash-free dry biomass, corrected for residual water, in ash-free dry biomass

w_{ash} weight of ash per total dry weight; mass fraction of ash in total dry biomass

w_C mass fraction of organic carbon in ash-free dry biomass; weight specific organic carbon

$w_C^{\ominus}$ $=w_C/w^{\ominus}$; mass fraction of organic carbon in ash-free dry biomass, corrected for residual water

${}_{ash}w_C$ mass fraction of (inorganic) carbon in ash

${}_{tot}w_C$ mass fraction of total carbon in total dry biomass

w_H mass fraction of hydrogen (organic and bound to residual water) in ash-free dry biomass

w_{H_2O} $=1-w^{\ominus}$; mass fraction of residual water in ${}_{af}W$

w_i mass fraction of substance i in ${}_{af}W$ (w_K, w_L, w_P)

w_N mass fraction of organic nitrogen in ${}_{af}W$

${}_{af}W$ ash-free dry biomass, dry weight minus ash weight [g]

${}_{d}W$ total dry weight [g]

x_{PN} mole (or mass) fraction of protein-nitrogen per total organic nitrogen

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