

***Paenibacillus thiaminolyticus* is not the cause of thiamine deficiency impeding lake trout (*Salvelinus namaycush*) recruitment in the Great Lakes**

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Abstract: Thiamine (vitamin B₁) deficiency is a global concern affecting wildlife, livestock, and humans. In Great Lakes salmonines, thiamine deficiency causes embryo mortality and is an impediment to restoration of native lake trout (*Salvelinus namaycush*) stocks. Thiamine deficiency in fish may result from a diet of prey with high levels of thiaminase I. The discoveries that the bacterial species *Paenibacillus thiaminolyticus* produces thiaminase I, is found in viscera of thiaminase-containing prey fish, and causes mortality when fed to lake trout in the laboratory provided circumstantial evidence implicating *P. thiaminolyticus*. This study quantified the contribution of *P. thiaminolyticus* to the total thiaminase I activity in multiple trophic levels of Great Lakes food webs. Unexpectedly, no relationship between thiaminase activity and either the amount of *P. thiaminolyticus* thiaminase I protein or the abundance of *P. thiaminolyticus* cells was found. These results demonstrate that *P. thiaminolyticus* is not the primary source of thiaminase activity affecting Great Lakes salmonines and calls into question the long-standing assumption that *P. thiaminolyticus* is the source of thiaminase in other wild and domestic animals.

Résumé : La carence en thiamine (vitamine B₁) est un problème d'envergure planétaire touchant tant les animaux sauvages et d'élevage que les humains. Chez les salmoninés des Grands Lacs, la carence en thiamine est cause de mortalité embryonnaire et entrave le rétablissement des stocks indigènes de touladi (*Salvelinus namaycush*). Une carence en thiamine chez les poissons peut découler d'une alimentation faite des proies avec de fortes teneurs en thiaminase I. Le fait que la bactérie *Paenibacillus thiaminolyticus* produit de la thiaminase I, qu'elle est présente dans les viscères de poissons proies contenant de la thiaminase et qu'elle cause une mortalité quand elle est donnée à manger à des touladis en laboratoire sont autant de découvertes offrant des preuves circonstancielles de l'implication de *P. thiaminolyticus*. L'étude a quantifié la contribution de *P. thiaminolyticus* à l'activité totale de la thiaminase I dans de multiples niveaux trophiques de réseaux trophiques des Grands Lacs. Contre toute attente, aucun lien n'a été observé entre l'activité de la thiaminase et la quantité de protéine thiaminase I de *P. thiaminolyticus* ou l'abondance de cellules de *P. thiaminolyticus*. Ces résultats démontrent que *P. thiaminolyticus* n'est pas la principale source d'activité de la thiaminase affectant les salmoninés des Grands Lacs et remet en question le postulat bien établi voulant que *P. thiaminolyticus* soit la source de la thiaminase dans d'autres animaux sauvages ou domestiques.

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Introduction

Thiamine (vitamin B₁) is an essential nutrient that is required for carbohydrate metabolism and neural function. Thiamine deficiency causes adverse effects in fish (Brown et al. 2005), wildlife (Balk et al. 2009), agriculturally important an-

imals (Green and Evans 1940; Edwin and Jackman 1970), and humans (Lonsdale 2006) and may be caused by insufficient intake of thiamine, enzymatic degradation of thiamine by thiaminase, or other factors that alter the physiological availability of thiamine. Thiamine may be degraded enzymatically by thiaminase I (thiamine pyrimidinylase, EC 2.5.1.2),

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which requires a nucleophilic co-substrate, or by thiaminase II (EC 3.5.99.2), which uses water as the nucleophile. Thiaminase II has a role in thiamine salvage (Jenkins et al. 2007), but the physiological role for thiaminase I has yet to be defined. Thiamine deficiency suspected to be induced by thiaminase I (hereafter thiaminase) can lead to mortality and has been reported in fish (Honeyfield et al. 2005), alligators (Honeyfield et al. 2008), chickens (Shintani 1956), foxes, mink, cats, ruminants, and humans (Evans 1975). However, identification of the ultimate source(s) of thiaminase enzymes in animals and their physiological function in vivo remains elusive (Riley and Evans 2008). Previous attempts to identify the specific organism(s) with genes encoding the thiaminase enzymes that may contribute to thiamine deficiency in animals suggested a role for microbes in the gut but were not conclusive (Edwin and Jackman 1970; Boyd and Walton 1977; Honeyfield et al. 2002). Identifying the source(s) of thiaminase in animals is essential for understanding its effects on animals of importance to agriculture and aquaculture. This investigation is also important for conservation biology because thiamine deficiency suspected to be induced by thiaminase is associated with mortality of wild animal populations, including lake trout (*Salvelinus namaycush*) (Fitzsimons et al. 1999), Atlantic salmon (*Salmo salar*) (Ketola et al. 2000; Michielsens et al. 2006), and the American alligator (*Alligator mississippiensis*) (Honeyfield et al. 2008), in ecosystems ranging from the Great Lakes to the Baltic Sea to Florida lakes and wetlands.

In the Laurentian Great Lakes of North America, lake trout, the historically dominant native apex predator, have shown severe declines in abundance followed by recruitment failure due to multiple stressors, including sea lamprey (*Petromyzon marinus*) predation, overfishing, contaminants, predation by non-native alewife (*Alosa pseudoharengus*) on eggs and embryos, and nearshore habitat degradation (Krueger and Ebener 2004; Tillitt et al. 2008). In recent decades, high mortality rates of lake trout embryos resulting from thiamine deficiency have been shown to be widespread and thus may undermine restoration efforts by contributing to recruitment failure (Fisher et al. 1996; Brown et al. 2005). Recognition of the potential role of thiamine deficiency in limiting lake trout recruitment is demonstrated in the recent "Guide for the rehabilitation of lake trout in Lake Michigan", which identified thiamine deficiency as the sole "impediment of special concern" (Bronte et al. 2008). Lake trout embryos with insufficient thiamine suffer mortality between hatch and exogenous feeding, and surviving embryos with low thiamine levels suffer impaired vision, reduced foraging ability, and reduced growth (Carvalho et al. 2009; Fitzsimons et al. 2009). The common and dominant prey items for lake trout and other salmonines in the Great Lakes contain sufficient levels of thiamine, and thus salmonines do not produce thiamine-deficient eggs based on ingestion of too little thiamine (Tillitt et al. 2005). At many locations, the favored prey species for salmonines >500 mm in length is the alewife, an invasive species that contains high thiaminase activity (Brown et al. 2005; Madenjian et al. 2006). The source of thiaminase activity found in alewife and other thiaminase-containing organisms has not been determined. Non-native dreissenid mussels

(zebra mussel, *Dreissena polymorpha*, and quagga mussel, *Dreissena bugensis*) also contain high thiaminase activity, although the impact of dreissenid mussel thiaminase on thiamine status in predators of dreissenids is not known (Tillitt et al. 2009).

The Gram-positive bacterial species *Paenibacillus thiaminolyticus* has a gene encoding a thiaminase I enzyme, and the thiaminolytic activity of the enzyme has been experimentally verified (Abe et al. 1987). The discovery that alewife collected from Lake Michigan contained *P. thiaminolyticus* in their viscera (Honeyfield et al. 2002) suggested *P. thiaminolyticus* as the source of thiaminase activity in alewife. Furthermore, lake trout fed a diet of alewife or a diet supplemented with *P. thiaminolyticus* culture developed thiamine deficiency that led to early life-stage mortality of embryos (Honeyfield et al. 2005). Despite this and other circumstantial evidence implicating *P. thiaminolyticus* as the source of thiaminase in aquatic and terrestrial animals, previous investigations specifically designed to identify the source of thiaminase have been inconclusive (Shintani 1956; Boyd and Walton 1977). Part of the reason for the lack of conclusive evidence in these studies results from reliance on culture-based techniques to quantify the amount of *P. thiaminolyticus* in samples.

The objective of this study was to determine whether *P. thiaminolyticus* is the primary source of thiaminase activity in fish viscera, zooplankton, and dreissenid mussels in the Great Lakes. Sampling was conducted at five sites with diverse food web structures and representing a range of severity of thiamine deficiency in lake trout. Immunoblotting and quantitative PCR methods were applied to determine the abundance of *P. thiaminolyticus* thiaminase and *P. thiaminolyticus* cells. We report the unexpected finding that thiaminase produced by *P. thiaminolyticus* is not a primary source of thiaminase activity in alewife or other components of Great Lakes food webs.

Materials and methods

Sample collection

Food web components (fish, zooplankton, and dreissenid mussels) were sampled at five sites in the Great Lakes, including Sturgeon Bay, Wisconsin, and Frankfort, Michigan (Lake Michigan); Ashland, Wisconsin (Lake Superior); Detroit, Michigan (Lake Huron); and Port Weller, Ontario (Niagara Bar in Lake Ontario). The sites chosen represented a variety of trophic structures resulting primarily from differences in the diversity of invasive species. Sampling was conducted from the US Geological Survey Research Vessels *Sturgeon*, *Kaho*, and *Kiyi* during three sampling periods: early spring (19 April), midsummer (mid-July – early August), and fall (mid-September – early October) of 2007. Samples were collected in nearshore (<18 m), mid-depth (30–60 m), and offshore (75–110 m) locations. The distribution of samples across species, sites, depth, and seasons can be found in Supplementary Tables S1 and S2¹.

Fish were sampled using bottom trawls and sorted by species. Up to 10 individual fish of each species were collected at each site for each season and each depth sampled. Zoo-

¹Supplementary data are available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/f2012-043>.

plankton were sampled using depth-integrated vertical tows of 153 and 53 μm nylon mesh nets starting 5 m above the substrate to exclude benthos. Samples were sorted in the field to remove larger organisms, including *Bythotrephes*, *Cercopagis*, and *Mysis*. Separate samples of the entire zooplankton community were fractionated by size by pouring the samples over a series of nylon mesh sieves with mesh sizes of 125, 53, and 25 μm (referred to as “bulk” samples). Dreissenid mussels were collected from bottom trawls. All collected samples for all taxa were placed in plastic bags and frozen immediately between slabs of dry ice.

For subsequent analyses, individual fish were lightly thawed on ice, all visceral tissue was removed, and the viscera and remaining carcass were immediately refrozen separately on dry ice. Previous studies have shown that thiaminase activity is stable in thawed, previously frozen alewife samples (Wright et al. 2005). Frozen fish viscera samples and other large samples (>0.6 g) were pulverized with dry ice in ceramic mortars and pestles. Once pulverized, the remaining dry ice in each powdered sample was allowed to sublimate first at $-20\text{ }^{\circ}\text{C}$, then at $-80\text{ }^{\circ}\text{C}$. The frozen powdered tissue was then randomly subsampled for thiaminase activity, quantitative polymerase chain reaction (Q-PCR) analysis, and Western blot analysis. Subsamples were weighed into 3.6 mL Nalgene cryovials (USA Scientific, Ocala, Florida) and stored at $-80\text{ }^{\circ}\text{C}$. All subsampling and weighing activities were carried out in a cold room at $4\text{ }^{\circ}\text{C}$ or $-20\text{ }^{\circ}\text{C}$ using a pre-equilibrated model AE163 analytical balance (Mettler Instruments Corporation, Hightstown, New Jersey). Plankton samples consisted of pools of many individuals, and the same single pool was subsampled for thiaminase activity, Q-PCR analysis, and Western blot analysis. Replicates for plankton samples were pools from separate tows. Dreissenid mussel shells were removed before analysis. Separate subsamples consisting of several individual mussels each were analyzed for thiaminase activity, Q-PCR analysis, and Western blot analysis. Therefore, dreissenid mussel data were averaged for each sampling event, which was defined as the unique combination of species, site, depth, and season.

Thiaminase activity

Thiaminase activity was determined in whole homogenates using a standard radiometric assay (Zajicek et al. 2005). Analyses were conducted on individual fish viscera or on pooled groups of organisms that were too small to be analyzed individually. Briefly, tissue samples were assayed directly as whole homogenate suspensions prepared and diluted in $0.1\text{ mol}\cdot\text{L}^{-1}$, pH 6.5, potassium phosphate buffer containing $0.1\text{ mol}\cdot\text{L}^{-1}$ NaCl. Aliquots of whole homogenates were combined with aliquots of co-substrate, nicotinic acid ($40\text{ mmol}\cdot\text{L}^{-1}$, pyridine-3-carboxylic acid, Sigma-Aldrich Corp., St. Louis, Missouri), radiometric substrate, thiazole-2- ^{14}C -thiamine (Amersham Pharmacia Biotech, Piscataway, New York), diluted with ^{12}C -thiamine (Sigma-Aldrich) to $0.075\text{ mmol}\cdot\text{L}^{-1}$ total thiamine (specific activity of $17.5\text{ disintegrations per minute (DPM)}\cdot\text{pmol}^{-1}$), and assayed at $37\text{ }^{\circ}\text{C}$ for 10 min. The assay temperature of $37\text{ }^{\circ}\text{C}$ was chosen to maximize detection of thiaminase activity (Zajicek et al. 2005). The enzyme reactions were stopped with aliquots of cold ethyl acetate (Optima, Fisher Scientific, Fairlawn, New Jersey). The ^{14}C -thiazole product of thiaminase activity was

partitioned into ethyl acetate and combined with Ecolume liquid scintillation cocktail (MP-Biochemicals, LLC, Solon Ohio), and the associated ^{14}C radioactivity was measured using a model LS 6500 liquid scintillation counter (Beckman Coulter, Brea, California). Measured DPMs were converted to specific activities in units of $\text{pmol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$. We analyzed all sample homogenates at a minimum of two dilutions and compared dilution-corrected results to verify linearity and the lack of matrix effects on measured thiaminase activities.

Western blot quantification of *P. thiaminolyticus* thiaminase protein

Thiaminase protein produced by *P. thiaminolyticus* was detected by immunoblotting using standard methods (Sambrook and Russell 2001). The primary antibody was a rabbit polyclonal antiserum made by Strategic Diagnostics Inc. (Newark, Delaware) to a partially purified His-tagged recombinant thiaminase derived from *P. thiaminolyticus* strain 8118 (Honeyfield et al. 2002). The secondary antibody was goat anti-rabbit alkaline phosphatase conjugate (Sigma-Aldrich Corp.).

Tissues were homogenized in potassium phosphate-buffered saline ($100\text{ mmol}\cdot\text{L}^{-1}$ NaCl, $83\text{ mmol}\cdot\text{L}^{-1}$ KH_2PO_4 , $17\text{ mmol}\cdot\text{L}^{-1}$ K_2HPO_4 , pH 6.5) containing protease inhibitors: $0.52\text{ mmol}\cdot\text{L}^{-1}$ 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), $0.4\text{ }\mu\text{mol}\cdot\text{L}^{-1}$ aprotinin, $10\text{ }\mu\text{mol}\cdot\text{L}^{-1}$ leupeptin, $20\text{ }\mu\text{mol}\cdot\text{L}^{-1}$ bestatin, $7.5\text{ }\mu\text{mol}\cdot\text{L}^{-1}$ pepstatin, and $7\text{ }\mu\text{mol}\cdot\text{L}^{-1}$ *trans*-epoxysuccinyl-L-leucylamido (4-guanidino)butane (E64). All reagents were supplied by Sigma-Aldrich unless otherwise specified. Soluble proteins were extracted from homogenized tissues with CellLytic B reagent containing $2.5\text{ kU}\cdot\text{mL}^{-1}$ rlysozyme (EMD Chemicals, Gibbstown, New Jersey) and $25\text{ U}\cdot\text{mL}^{-1}$ benzonase nuclease (EMD Chemicals). The protease inhibitor cocktail was tested for effectiveness in a subset of alewife viscera samples using an azocasein protease assay as described (Tomarelli et al. 1949). Total soluble proteins were separated by molecular mass via discontinuous sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE). Separated proteins were transferred from gels to nitrocellulose membranes. Transferred proteins were visualized with MemCode reversible protein stain (Thermo Fisher Scientific, Rockford, Illinois). Antibody-bound protein bands were visualized by chemiluminescence using CDP-Star AP substrate with Nitro-Block II signal enhancer (EMD Chemicals) and quantified with a ChemiImager 5500 (Alpha Innotech, San Leandro, California). Duplicate homogenates were run for 10% of samples. Recombinant thiaminase protein at two concentrations and one thiaminase-spiked food web sample were included on each gel (Fig. S1¹).

The Western blot methods successfully recovered *P. thiaminolyticus* thiaminase protein from the sample matrix, and densitometry measurements gave semiquantitative estimates of amounts of *P. thiaminolyticus* thiaminase protein (Fig. S1¹). Thiaminase protein encoded by *P. thiaminolyticus* was spiked into food web samples either as recombinant purified protein or as native intracellular protein in *P. thiaminolyticus* cells and was recovered at approximately the expected levels (Fig. S1¹). The limit of detection for this assay was gel-specific and averaged 0.85 micrograms of *P. thiaminolyticus* thiaminase protein per gram of tissue.

Q-PCR quantification of genetic markers for *P. thiaminolyticus*

Genetic markers were used to determine the abundance of *P. thiaminolyticus* cells as previously described (Richter et al. 2009). Quantitative PCR assays for total bacteria and the *P. thiaminolyticus* 16S rRNA gene were performed. Assays for the *P. thiaminolyticus* 16S rRNA gene used pre-amplification to maximize sensitivity. Reported *P. thiaminolyticus* cell numbers are based on the *P. thiaminolyticus* 16S rRNA gene assay of a standard curve of eight food web samples spiked with between 5.5×10^2 and 5.5×10^6 *P. thiaminolyticus* cells per gram of tissue (Fig. S2¹).

Total DNA was isolated from samples using the DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, California) according to the manufacturer's instructions for Gram-positive bacteria or the AGP245T Tissue DNA extraction kit (AutoGen, Holliston, Massachusetts) according to the manufacturer's instructions, with the addition of a 30 min predigestion at room temperature with 7.5 U- μL^{-1} rlysozyme (EMD Chemicals). The Q-PCR methods recovered *P. thiaminolyticus* DNA from the sample matrix in a quantitative fashion (Fig. S2¹). Reported total bacterial cell numbers are based on the previously reported linear correlation of the universal bacterial 16S rRNA gene assay with known cell densities of pure cultures of four bacterial species (Richter et al. 2009). Based on the Q-PCR limit of detection of one copy per reaction and loading the equivalent of 0.001 g tissue per reaction, the limit of detection for the *P. thiaminolyticus* 16S rRNA gene assay was 17 *P. thiaminolyticus* cells- g^{-1} . Increased variation is expected near the limit of detection because of stochastic effects on the number of copies of the target sequence introduced to each reaction.

Bacterial culture

Thiaminase activity, *P. thiaminolyticus* thiaminase protein, and colony-forming units (CFU) were measured in pure cultures of *P. thiaminolyticus* strain 8118 (Honeyfield et al. 2002). The thiaminase activity and Western blot assays were performed on whole culture medium to measure both intracellular and excreted thiaminase. The results of this experiment were used to estimate the expected number of *P. thiaminolyticus* cells and amount of *P. thiaminolyticus* thiaminase protein associated with measured levels of thiaminase activity. Replicate cultures were grown in Terrific Broth (MO BIO Laboratories, Carlsbad, California) in a shaking incubator at 37 °C and were harvested after 80 h of culture. These culture conditions were optimized for the maximum thiaminase activity per volume of *P. thiaminolyticus* culture. For CFU counts, dilutions of bacterial cultures were plated onto Luria-Bertani agar plates (Sambrook and Russell 2001).

Data analysis

Statistical analyses were conducted in SigmaPlot (Systat Software, Chicago, Illinois) or R (R Development Core Team 2008). The association between thiaminase activity and either the amount of *P. thiaminolyticus* thiaminase protein (as measured by densitometry of Western blots) or the abundance of *P. thiaminolyticus* cells (as measured by Q-PCR of the 16S rRNA gene) was quantified using a Spearman rank correlation. The Spearman rank correlation was chosen because variables were censored at the detection limit

for each assay (20 pmol- $\text{g}^{-1}\cdot\text{min}^{-1}$ for the thiaminase activity assay, 0.85 $\mu\text{g}\cdot\text{g}^{-1}$ for the thiaminase protein assay, and 17 *P. thiaminolyticus* cells- g^{-1} for the Q-PCR assay) and because relationships between the predictor and response were often nonlinear. All reported *p* values are for two-tailed tests.

Results

Western blot quantification of *P. thiaminolyticus* thiaminase protein

Overall, the amount of *P. thiaminolyticus* thiaminase protein measured was not positively related to the level of thiaminase activity measured in samples of fish viscera, zooplankton, or dreissenid mussels, as would be expected if *P. thiaminolyticus* thiaminase was the predominant source of thiaminase activity (Fig. 1; Supplemental Tables S3–S6¹). No positive relationship was found between the amount of *P. thiaminolyticus* thiaminase protein and thiaminase activity for all individual species, sites, and seasons examined (Tables S3–S6¹). Although dreissenid mussels were grouped by sampling event rather than being analyzed as individual samples, the same trends were observed in dreissenid mussels as in fish viscera and zooplankton samples; we found no positive relationship between thiaminase activity and *P. thiaminolyticus* thiaminase protein, and some sampling events had high average thiaminase activity but no detectable *P. thiaminolyticus* thiaminase protein. Our comparison of *P. thiaminolyticus* thiaminase protein quantity and thiaminase activity contradicts the hypothesis that *P. thiaminolyticus* thiaminase is the source of thiaminase activity in Great Lakes food web samples.

Representative protein blots and Western blots for *P. thiaminolyticus* thiaminase are shown (Figs. 1a, 1c, 1e), and estimated *P. thiaminolyticus* thiaminase protein for all samples is shown (Figs. 1b, 1d, 1f). The dashed lines (Figs. 1b, 1d, 1f) indicate the expected relationship between thiaminase activity and *P. thiaminolyticus* thiaminase protein based on culture experiments (Table 1). Points above the dashed line have greater thiaminase activity than can be explained by the estimated amount of *P. thiaminolyticus* thiaminase protein. Many samples with high thiaminase activity had undetectable levels of *P. thiaminolyticus* thiaminase protein (Figs. 1b and 1f), and therefore *P. thiaminolyticus* thiaminase cannot serve as the source of thiaminase activity in these samples. At the average limit of detection of the *P. thiaminolyticus* thiaminase protein assay of 0.85 $\mu\text{g}\cdot\text{g}^{-1}$ (dotted lines, Figs. 1b, 1d, 1f), the predicted thiaminase activity based on *P. thiaminolyticus* laboratory cultures was 8000 pmol- $\text{g}^{-1}\cdot\text{min}^{-1}$ (Table 1). Thus, if *P. thiaminolyticus* is the major source of thiaminase activity, samples with thiaminase activity greater than 8000 pmol- $\text{g}^{-1}\cdot\text{min}^{-1}$ would be expected to have detectable *P. thiaminolyticus* thiaminase protein in the Western blot assay, and yet many samples containing more than 8000 pmol- $\text{g}^{-1}\cdot\text{min}^{-1}$ thiaminase activity had undetectable levels of *P. thiaminolyticus* thiaminase protein. In addition, some samples that scored positive for *P. thiaminolyticus* thiaminase protein had thiaminase activity levels below the activity predicted at the limit of detection of the Western blot assay, suggesting that some *P. thiaminolyticus* thiaminase protein measurements were false positives.

False positives in the Western blots from nonspecific bind-

Fig. 1. Semiquantitative Western blot of thiaminase produced by *P. thiaminolyticus* did not correlate with thiaminase activity of fish viscera (*a* and *b*), zooplankton (*c* and *d*), and dreissenid mussels (*e* and *f*). (*a*, *c*, and *e*) The upper halves of the panels are stained for all proteins, and the bottom halves of the panels are Western blots for *P. thiaminolyticus* thiaminase. In each panel, lane 1 is a thiaminase standard and lanes 2–5 are representative samples. The arrows indicate the expected molecular masses of thiaminase with and without its N-terminal signal sequence. The expected thiaminase activity of the lane 1 standard is 94 000 pmol·g⁻¹·min⁻¹. See text for explanations of the lines in panels (*b*), (*d*), and (*f*).

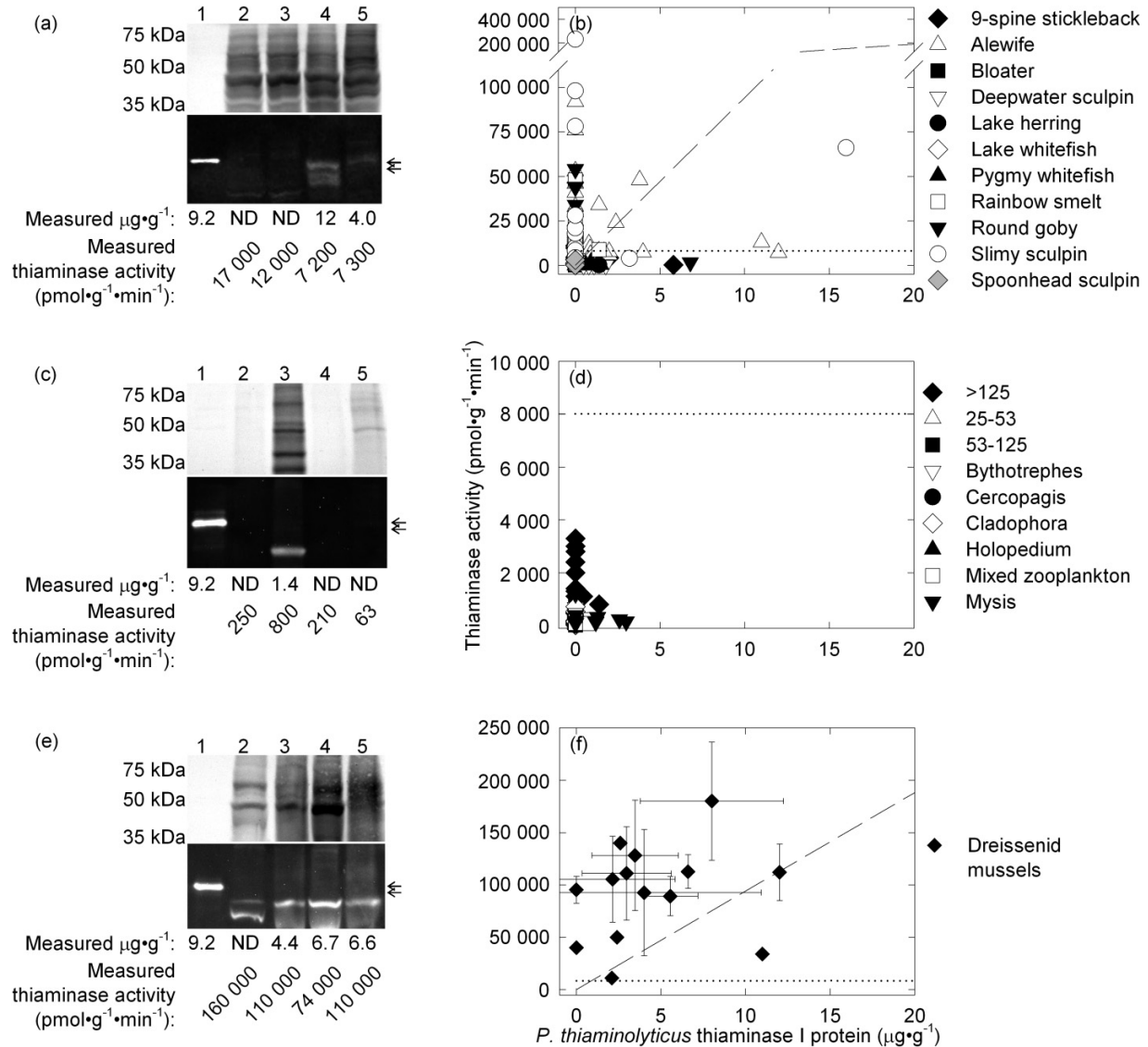


Table 1. Thiaminase activity, thiaminase protein, and colony-forming units (CFU) produced by *P. thiaminolyticus* under laboratory culture conditions.

Culture	Thiaminase activity (pmol·mL ⁻¹ ·min ⁻¹)	<i>P. thiaminolyticus</i> thiaminase (μg·mL ⁻¹)	CFU·mL ⁻¹
Pt-1	210 000±21 000	21±11	1.1×10 ⁸ ±5.4×10 ⁷
Pt-2	200 000±26 000	19±11	1.2×10 ⁸ ±8.1×10 ⁷
Pt-3	200 000±27 000	26±15	2.1×10 ⁸ ±1.0×10 ⁸
Average	200 000	22	1.5×10 ⁸

ing are not unexpected, given the complexity and diversity of the sample set. Nonspecific binding of the anti-thiaminase antibodies was observed in many food web samples (Fig. S1¹). In some fish viscera and plankton samples, a nonspecific band was observed at approximately 35 kDa in the Western blots (Figs. S1a and S1b¹). This band was not in-

cluded in the densitometry results because it clearly migrates differently from *P. thiaminolyticus* thiaminase. Some dreissenid mussel samples contained a protein that bound the anti-*P. thiaminolyticus* thiaminase antibodies and that was near the molecular mass of mature *P. thiaminolyticus* thiaminase (Fig. S1c¹). However, when *P. thiaminolyticus* cells were

spiked into dreissenid mussel samples containing this protein, three distinct bands were visible, corresponding to the full-length *P. thiaminolyticus* thiaminase protein containing the N-terminal signal sequence for extracellular secretion, mature *P. thiaminolyticus* thiaminase protein with the signal sequence cleaved, and the dreissenid cross-reacting protein (Fig. S1c¹). Although the dreissenid protein may not be *P. thiaminolyticus* thiaminase, we included it in densitometry measurements because it migrates close to mature *P. thiaminolyticus* thiaminase. This may have led to false positive *P. thiaminolyticus* thiaminase protein detections in dreissenid mussel samples.

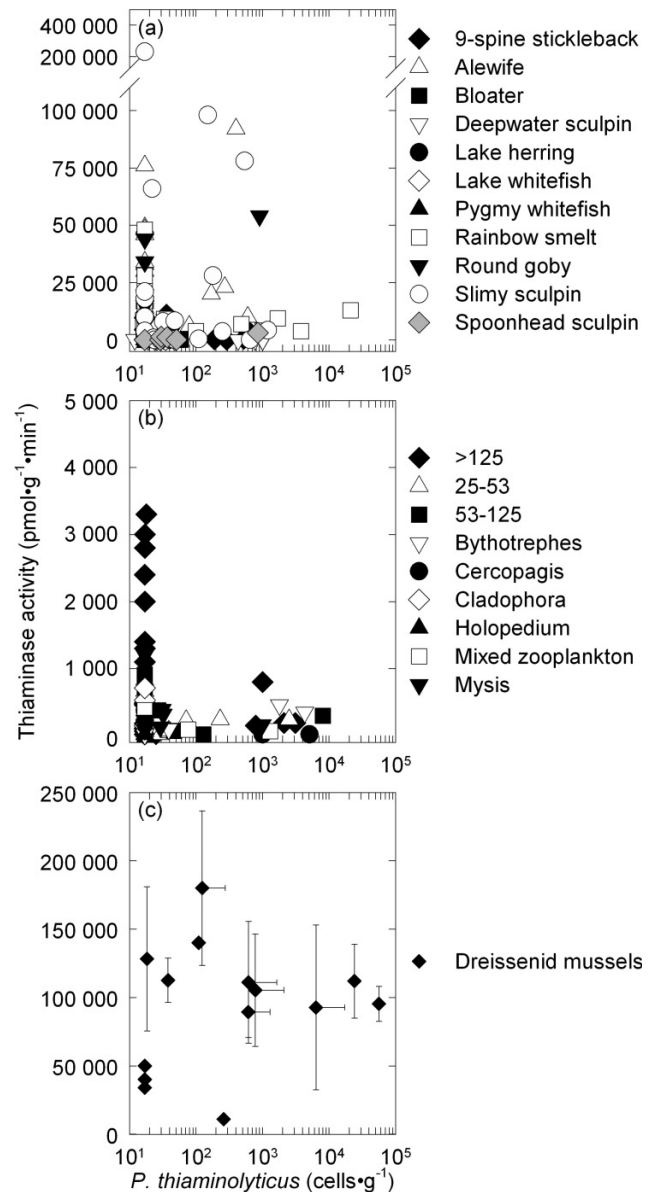
The cross-reacting protein observed in dreissenid mussels raises the possibility that a protein similar to *P. thiaminolyticus* thiaminase contributed to the thiaminase activity observed in dreissenids. The cross-reacting protein was slightly smaller than *P. thiaminolyticus* thiaminase and did not show a significant positive relationship with abundance of *P. thiaminolyticus* cells (data not shown). It is possible that the cross-reacting protein was a modified form of *P. thiaminolyticus* thiaminase or a different thiaminase protein from a related species of bacteria. However, this protein cannot be the major source of thiaminase activity in dreissenids because it was not present in all samples with high activity and was not positively related to thiaminase activity.

A few fish viscera and dreissenid mussel samples had estimated *P. thiaminolyticus* thiaminase protein levels close to the levels expected based on their thiaminase activity (Figs. 1b and 1f). However, none of these samples had populations of *P. thiaminolyticus* cells great enough to produce the measured thiaminase activity (see Q-PCR results below). One hypothesis is that in these samples, thiaminase protein secreted from *P. thiaminolyticus* was accumulated independently of the bacteria themselves. Another possibility is that these samples are false positives; this is not unlikely given the known vulnerability of the Western blot assay to nonspecific protein binding, as discussed above. The number of samples in which *P. thiaminolyticus* thiaminase protein levels were close to the levels expected based on their thiaminase activity was a very small proportion of the total number of samples analyzed.

Q-PCR quantification of genetic markers for *P. thiaminolyticus*

A quantitative PCR assay specific for the 16S rRNA gene of *P. thiaminolyticus* was used to measure the abundance of *P. thiaminolyticus* cells in each food web sample (Fig. 2). The abundance of *P. thiaminolyticus* cells was not positively related to thiaminase activity in fish viscera, zooplankton, or dreissenid mussels, as would be expected if *P. thiaminolyticus* was the source of their thiaminase activity (Fig. 2; Tables S3–S6¹). The lack of a positive relationship between the abundance of *P. thiaminolyticus* cells and thiaminase activity occurred for all individual species, sites, and seasons examined, with the single exception of bulk zooplankton fractions collected at Ashland, Wisconsin ($n = 6$; Table S4¹). The bulk zooplankton fractions from Ashland had thiaminase activity near the limit of detection, resulting in little overall contrast in thiaminase activity. Many individual samples with high thiaminase activity had undetectable *P. thiaminolyticus* cells, and conversely, many samples with detectable *P. thiaminolyti-*

Fig. 2. Abundance of *P. thiaminolyticus* cells did not correlate with thiaminase activity in individual samples of fish viscera (a), pooled samples of zooplankton (b), and pooled samples of dreissenid mussels (c). Note log scale on x axis.



cus cells had low thiaminase activity (Fig. 2). Specifically, at the limit of detection of the thiaminase activity assay of 20 pmol·g⁻¹·min⁻¹, *P. thiaminolyticus* laboratory culture experiments (Table 1) predicted a *P. thiaminolyticus* cell abundance of 1.4×10^4 cells·g⁻¹. Thus, if *P. thiaminolyticus* is the major source of thiaminase activity, samples with detectable thiaminase activity would be expected to have *P. thiaminolyticus* populations greater than 1.4×10^4 cells·g⁻¹, and yet only 4 out of 253 thiaminase-positive samples had *P. thiaminolyticus* cell abundances that high (Fig. 2). Even for those four samples, thiaminase activity was orders of magnitude higher than could be accounted for by the measured abundance of *P. thiaminolyticus*.

Of the 308 total food web samples assayed (Tables S1 and S2¹), 253 had detectable levels of thiaminase activity, and no *P. thiaminolyticus* cells were detected in 163 of the 253

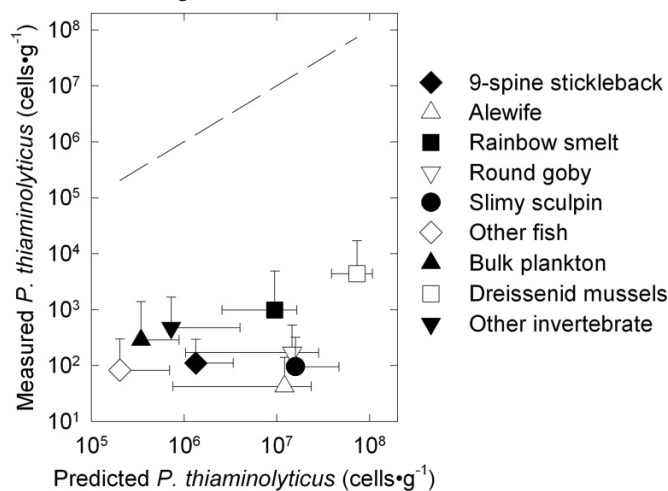
thiaminase-positive samples. Thus, *P. thiaminolyticus* cells were only detected in 36% of all thiaminase-containing samples. For alewife alone, thiaminase activity was detected in each of the 56 fish viscera sampled, and *P. thiaminolyticus* cells were detected in only 5 of the 56 (9%). These findings are consistent with that of a previous study in which *P. thiaminolyticus* was cultured from 25% of alewife examined, all of which contained thiaminase activity (Honeyfield et al. 2002). The universal bacterial 16S rRNA gene assay was able to detect bacteria in all samples (Fig. S3¹). Thus, the DNA isolation procedure captured bacterial DNA, and the Q-PCR assay was functional in the food web samples. Furthermore, *P. thiaminolyticus* cells spiked into food web samples were quantitatively recovered by the *P. thiaminolyticus* 16S rRNA gene assay at levels as low as 550 cells·g⁻¹, the lowest level tested (Fig. S2¹). In summary, the failure to detect *P. thiaminolyticus* in many food web samples, including those with high thiaminase activity, was not due to technical limitations of the Q-PCR assay but was due to the absence of *P. thiaminolyticus* cells. Furthermore, given the data from culture experiments (Table 1), the number of *P. thiaminolyticus* cells expected to produce the thiaminase activity observed in Great Lakes food web samples was orders of magnitude higher than the abundances of *P. thiaminolyticus* actually observed in food web samples (Fig. 3).

Discussion

Our results indicate that *P. thiaminolyticus* is not a major source of thiaminase activity in any sampled component of the Great Lakes food web. This unexpected conclusion is supported by two lines of evidence. The first line of evidence is the lack of positive relationships between thiaminase activity and either the amount of *P. thiaminolyticus* thiaminase protein or the number of *P. thiaminolyticus* cells in any food web item examined. Consistently, across every taxa, no relationship or a negative relationship existed between thiaminase activity and the amount of *P. thiaminolyticus* thiaminase protein or the number of *P. thiaminolyticus* cells. The second line of evidence is that both the amount of *P. thiaminolyticus* thiaminase protein detected and the number of *P. thiaminolyticus* cells detected were much smaller than would be required to produce the measured thiaminase activity based on laboratory culture experiments. Many food web samples with high thiaminase activity had undetectable levels of *P. thiaminolyticus* thiaminase protein and *P. thiaminolyticus* cells. Because the *P. thiaminolyticus* thiaminase protein is secreted and may accumulate in samples even if the abundance of *P. thiaminolyticus* cells was low, we measured both *P. thiaminolyticus* thiaminase protein and *P. thiaminolyticus* cells. These two independent assays consistently found no association between thiaminase activity and *P. thiaminolyticus*.

Previous research has focused on *P. thiaminolyticus* as the likely source of thiaminase activity in aquatic and terrestrial animals, including ruminants, birds, and fishes (Shintani 1956; Boyd and Walton 1977; Honeyfield et al. 2002). Although *P. thiaminolyticus* has been isolated from thiaminase-containing animals (Shintani 1956; Honeyfield et al. 2002), biochemical properties of purified thiaminases from thiaminase-containing animals have been found to be inconsistent with those of *P. thiaminolyticus* (Fujita 1954;

Fig. 3. Measured abundance of *P. thiaminolyticus* cells compared with abundance predicted from thiaminase activity. Dashed line indicates expected relationship if *P. thiaminolyticus* is the source of the observed thiaminase activity. Error bars are standard deviations. Error bars crossing 0 are not shown.



Boyd and Walton 1977; Zajicek et al. 2009), leaving open the question of whether *P. thiaminolyticus* was the source of thiaminase in thiaminase-containing animals. By showing that thiaminase activity is not the result of *P. thiaminolyticus*, we have eliminated the heretofore most often cited answer to the question of the ultimate source of thiaminase activity in aquatic animals. Our results also raise the possibility that *P. thiaminolyticus* is not the source of thiaminase activity in terrestrial wildlife and livestock affected by thiaminase-induced thiamine deficiency.

The true source of thiaminase activity in aquatic ecosystems remains elusive. Potential sources that merit further investigation include non-*P. thiaminolyticus* bacteria, de novo synthesis by lower trophic-level food items such as zooplankton or mussels, and de novo synthesis of thiaminase by fishes (Deolalkar and Sohoni 1954; Boš and Kozik 2000; Nishimune et al. 2008). Many potential bacterial sources of thiaminase activity exist, and comparing the composition of the bacterial flora in fish viscera with and without thiaminase activity would be useful for determining if particular bacterial species are associated with increased thiaminase activity. Putative thiaminase enzymes have been identified in genomic sequences from several species of bacteria, including *Clostridium botulinum* (GenBank accession No. ADF98595; 69% amino acid sequence similarity to *P. thiaminolyticus* thiaminase), a bacterial species for which some information about the biochemical properties of thiaminase is available (Fujita 1954).

Non-bacterial sources of thiaminase constitute additional potential sources of thiaminase activity. Species-specific pH profiles for thiaminase activity from different prey fish suggest multiple, species-specific sources of thiaminase and are consistent with the possibility of de novo thiaminase synthesis by fish (Deolalkar and Sohoni 1954; Zajicek et al. 2009). Several studies suggest an internal regulatory mechanism for thiaminase activity in fish that would also be consistent with de novo thiaminase production; factors reported to influence thiaminase activity in prey fish are diet, stress from being

held in the laboratory, and pathogen challenge by a bacterial species that does not contain thiaminase activity (Lepak et al. 2008; Wistbacka et al. 2009; Honeyfield et al. 2010). Furthermore, putative thiaminase enzymes purified from two fish species showed no amino acid sequence homology to bacterial enzymes or to each other, consistent with species-specific sources of thiaminase activity (Boś and Kozik 2000; Nishimune et al. 2008).

Mortality resulting from thiamine deficiency suspected to be induced by thiaminase has been identified as an impediment of special concern for the rehabilitation of lake trout populations (Bronte et al. 2008). In the Baltic Sea, rehabilitation of Atlantic salmon populations is also impaired by a thiamine deficiency syndrome known as M74 suspected to be induced by thiaminase (Norrgren et al. 1998). In both the Great Lakes and the Baltic Sea, variation in thiaminase activity in prey fish across species, locations, and seasons exists, but the underlying reasons for the variation are currently unknown (Tillitt et al. 2005). Identifying the source of thiaminase activity is a necessary prerequisite to understanding the mechanisms regulating thiaminase production in fishes. Once the mechanisms regulating thiaminase activity in fishes are understood, these mechanisms may be exploited by managers to promote conditions that decrease thiaminase activity in fishes that serve as prey for lake trout or to minimize exposure of stocked lake trout to thiaminase activity in prey fishes.

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