UNDERSTANDING THE EVIDENCE *(for reference only)*

Some Evidence cards may need a brief explanation on **isotope ratio data**. When you see this symbol, refer back to this card.

What does isotope ratio data (values) for carbon, nitrogen, and sulfur tell us?

- The **flow of energy** through a food web, i.e. what animals are eating.
- That different geological and biological processes lead to different isotope ratios which can be used to **determine the source and track movement** of carbon-containing compounds in the environment.
  - For example, a carbon isotope ratio is a measure of the relative amounts of carbon-12 (the most abundant isotope) and carbon-13 (a heavier isotope) within a substance that contains carbon. Lower isotope values indicate lower proportions of the heavier isotope. The same logic applies for nitrogen and sulfur isotope ratios.

UNDERSTANDING THE EVIDENCE *(for reference only)*

Some Evidence cards may need a brief explanation on **genetic** analyses. When you see this symbol, refer back to this card.

Lim and colleagues (2022) collected the most recent evidence on methane ice worms by analyzing **metagenomes**, *complete sequencing of the genetic material extracted directly* from the worms' gut and tissue samples.

- Analyzing RNA sequences allows scientists to investigate the **evolutionary relationships and classification of species** that may be only distantly related.
- The ability to construct full sequences from fragments allows scientists to **detect the presence of specific organisms** without directly isolating and culturing those organisms.

EVIDENCE

Becker and colleagues **(2013)** analyzed isotope ratios in animals from multiple seep communities in the Gulf of America.

Ratios for methane ice worms varied greatly.

- **Carbon** isotope value range:
  -63 to -29 parts per thousand
- **Nitrogen** isotope value range:
  -6 to 5 parts per thousand
- **Sulfur** isotope value range:
  -21 to 0.7 parts per thousand

EVIDENCE

Examination of internal tissues through light and transmission electron microscopes showed only scattered bacteria in the gut. (Fisher and colleagues, 2000)

EVIDENCE

Fisher and colleagues (2000) found that the carbon isotope values measured in the methane ice worms were approximately -24 parts per thousand.

This is intermediate between the ratio found in the methane hydrate (-48 parts per thousand) and the ratio typically found in seawater (0 parts per thousand).

EVIDENCE

Fisher and colleagues (2000) found that the nitrogen isotope ratio values measured in methane ice worms ranged from 5.3 to 6.3 parts per thousand.

EVIDENCE

Fisher and colleagues (2000) found that the sulfur isotope ratio values in two methane ice worms were 1.9 and 3.6 parts per thousand.

EVIDENCE

Fisher and colleagues (2000) used a scanning electron microscope to examine the external surface of two methane ice worms and found “no external symbionts.”

EVIDENCE

Gut contents removed from methane ice worms consisted of clear liquid with black particulates.

Environmental exposure to hydrocarbons was indicated by a petroleum smell emanating from the worm gut when pierced during dissection. (Lim and colleagues, 2022).

EVIDENCE

Lim and colleagues (2022) determined the RNA genome (all the genetic material in an organism) sequences for all bacterial species found in the gut contents from two methane ice worms.

86% of these sequences were identified as ***Sulfurospirillum species*** (bacterial species known to metabolize sulfur-containing compounds.)

Species that metabolize methane were rare, and there was little evidence of bacterial symbiosis outside the gut.

EVIDENCE

Lim and colleagues (2022) found that some bacteria identified in the gut of the methane ice worm have genes that would allow them to synthesize certain B vitamins and amino acids.

EVIDENCE

Observation and dissection of the methane ice worms revealed that they have a functional digestive tract. Freshly collected worms produced feces for 24 hours. (Fisher and colleagues, 2000).

EVIDENCE

Only prokaryotes are known to use methane and sulfide gases as energy sources or to metabolize crude oil. (Fisher and colleagues, 2000)

EVIDENCE

Pile and Young (2006) found that in laboratory trials, methane ice worm larvae significantly reduced the growth rates of microscopic plankton, including **heterotrophic bacteria, autotrophic cyanobacteria**, and **phototrophic eukaryotes** that were less than $<3\mu$ m(micrometers) in size.

EVIDENCE

Samples collected from the surface of the hydrate revealed “an abundant and varied population of bacteria.” (Fisher and colleagues, 2000)

EVIDENCE

The *Sulfurospirillum* species identified by Lim and colleagues (2022) through ***genomic** analyses were genetically different from the free-living species that have been found previously.

Study of the complete set of DNA (including all of its genes) in a person or other organism.

EVIDENCE

When Fisher and colleagues (2000) observed and dissected the methane ice worms, they did not find any hypertrophied (enlarged) tissues.

REASONING

Analysis of animals living both at and away from hydrothermal vents indicates that nitrogen isotope ratios for vent animals is generally <10 parts per thousand, while all non-vent animals are generally >10 parts per thousand.

Animals that live at vents are presumed to either host symbiotic bacteria or consume organisms that do.

REASONING

Animals are known to metabolize the four major groups of macromolecules:

- carbohydrates
- proteins
- lipids
- nucleic acid

An animal that could directly metabolize methane, sulfide gas, or the hydrocarbons found in crude oil would be a major scientific discovery.

REASONING

Average isotope ratio values for sulfate compounds found in ocean water are near 20 parts per thousand. In marine food webs based on photosynthetic plankton, sulfur isotope ratios typically range from 15 to 20 parts per thousand.

Bacteria that use sulfur compounds as an energy source can have sulfur isotope ratio values as low as -30 parts per thousand.

Tube worms that live at seeps and are known to have a symbiotic relationship with sulfur-metabolizing bacteria are known to have sulfur isotope ratio values that range from -20 to 5 parts per thousand.

Animals tend to reflect the sulfur isotope ratios of their food sources (Kennicut II et al., 1992).

REASONING

Bacteria serving as internal symbionts would be expected to be observed in large quantities somewhere within the host organism, often in the digestive tract.

For example, a large and characteristic microbial community can be found in the digestive tracts of ruminant animals like cows.

REASONING

Black particulates found in a deep sea animal's gut are likely to contain sulfur. Similar material has been found in the digestive tract of juvenile hydrothermal vent shrimp and was believed to be a nutrition source for these juveniles before they switch to a mainly ***epibiont**-based diet in their adult forms.

**Organism that lives on the surface of another living organism*

REASONING

External symbionts are organisms that live on the surface of other organisms in a dependent relationship. These symbionts may act as parasites or provide the host organism with protection.

Alternately, external symbionts may provide the host with nutritional resources by metabolizing compounds that the host cannot use directly.

REASONING

Feeding experiments are needed to definitively determine an organism's food sources. However, genomic studies can establish that bacteria have an important nutritional role, either as food sources or as digestive symbionts in the gut.

High abundances of *Sulfurospirillum* species were also reported in the gut of the hydrothermal vent crab *Austinograea* species, which is also known to feed on bacteria.

REASONING

Hypertrophied, or enlarged, tissues can be observed in an organism that is hosting internal symbionts. This occurs when large populations of the symbiont are present in a specific tissue.

For example, some beetles have enlarged sacs in their digestive system that host bacterial symbionts. Root nodules holding nitrogen-fixing bacteria in legume plants are another example.

REASONING

If methane ice worms get their nutrition from ***methanotrophic bacteria** that were metabolizing the methane hydrate directly, then their carbon isotope ratio values would be expected to be similar to the ratio found in the methane hydrate.

prokaryotes that metabolize methane as their source of carbon and chemical energy.

If the worms were feeding on bacteria or other organisms that had drifted from the surrounding seawater, then their carbon isotope ratio values would be expected to be close to 0.

Values found in the methane ice worms are within the range found in giant tubeworms that live at hydrothermal vents and contain sulfur-metabolizing symbionts.

REASONING

Large variation in the isotope ratio values for an animal species indicates large variability in the isotope compositions of the microbes on which they feed. This suggests they do not specialize on a single type of bacteria.

REASONING

Reduction in growth rate of microorganisms when grown in the presence of a possible predator indicates that the larger organism is in fact ingesting the microorganisms.

Evidence that a larval stage of an organism feeds on a particular food source indicates it is possible the adult stage also feeds on that same food source. However, adults may also use different food sources than larva of the same species.

REASONING

Supplying B vitamins and amino acids that cannot be synthesized by the host is a common way that symbionts contribute to the nutrition of the host organism.

REASONING

The behavior of the methane ice worms may contribute to the growth of bacteria near the surface of the hydrates.

Small water currents caused by movement of the worms' appendages may increase the breakdown of the hydrate, releasing methane and sulfide gasses into the surrounding water. This could increase the supply of oxygen needed by the bacteria to grow. This may also form the depressions in which the worms live.

REASONING

Sulfurospirillum species found in the gut of the methane ice worms do not match previously identified species, indicating these new species may not live in the general environment.

This supports the idea that *Sulfurospirillum* species act as intestinal symbionts in methane ice worms.

This is an important finding that needs to be verified because it represents new information.

REASONING

Unlike most animals, tube worms collected from deep sea thermal vent communities have no digestive tract, mouth, or anus. This is definitive evidence that they must rely on symbiotic bacteria living inside the tissues of the tube worms that provide them with energy and organic compounds.

An animal that has a functional digestive tract may ingest food, rely on internal symbionts, or both.